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SOME OBSERVATIONS ON GASTRIC SECRETION AND ITS RELATIONSHIP TO THE URINARY REACTION.

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THIS article embodies a study of the changes that occur in the urinary acidity with observations on the gastric secretion over a corresponding period. Evidence is brought forward to show that the urinary changes may possibly afford additional information in the diagnosis of certain gastric disorders.

Bence-Jones (1845) was the first to describe an increased alkalinity in the urine following a meal. In his publication (1845) he states that Dr. Andrewes, of Belfast, also observing the alkalinity following a morning meal, "was led to observe the urine of about 15 students in good health immediately after it was voided about noon. He found it to be alkaline in about two-thirds of the cases. Whether the tendency to alkalescent urine may, as Dr. Andrewes suggests, be connected with the immunity enjoyed by the inhabitants of his district from calculous affections, or whether alkalescence at this period of the day is far more general everywhere than has been supposed, future observation must determine." Bence-Jones ultimately concludes that the alkalinity does not depend on the nature of the food, but that the quantity of acid poured into the stomach probably sets free alkali sufficient in some cases to make the urine alkaline. He found the change to be more constant in persons suffering from "indigestion." Hofmann in 1898 stated that if the secretion of HCl was arrested the variation in the reaction of the urine did not occur. He observed that the acidity of the urine was not diminished after food in a case that had undergone total extirpation of the stomach (quoted by Dixon-Mann, 1905). Quincke (1884) suggested that the urinary changes were not in any way connected with the gastric secretion, but were diurnal changes. Leathes (1919) explained the alkaline tide, which he states is quite inconstant, by changes in respiration that occur on waking, while Campbell (1920) showed that the alkaline tide occurs regularly after the morning meal, and that it can be attributed in part at least to the secretion of HCl. Fiske (1921) came to a similar conclusion and emphasized the importance of the diet: a meal rich in phosphates would conceivably result in an increase of the pH of the urine

without any parallel change in titratable acidity. Adler (1919) studied the urinary changes in cases of anacidity and hypoacidity. Dodds (1923) estimated the A + N value, *i. e.* the titratable acidity per hour plus ammonia as c.c. N/100 alkali per hour, and the pH of the urine of persons known to have acid secretion in the stomach, and observed the changes following breakfast and lunch, and also the effects of starvation. He found that a decrease in the excretion of acid and ammonia followed gastric secretion, and that such changes were absent in starving controls. "The most noticeable point was that the pH of the starvation curves was found to be remarkably constant, whilst in the feeding subjects there was extreme variation in the figures." Watson (1923) criticizes the view that alkalinity of the urine is associated with the secretion of acid in the stomach, as he found the alkaline tide present in one case before water had been given by mouth, and in another case in which a tide was observed there was little free HCl present in the stomach at that period. Elizabeth Rannenberg (1926) recently published a comprehensive paper on the variations of the H-ion concentration of urine during the day. She attributes the change to the relationship of the mono- and di-sodium phosphates in solution. The only two conditions found in which no alkaline tide occurred were persistent starvation over a number of days and the persistent administration ot fatty meals. This work of Rannenberg is interesting in that it shows that, although a patient may not have his breakfast, he still according to habit secretes a sufficiency of HCl to alter the urinary reaction. But if, as she showed, a person misses his breakfast every morning for about a week, then no change occurs in the urine over the later period, although on the first day a definite alkaline tide would be obtained. It is a well-known fact that fats are strong inhibitors of gastric secretion, and although Rannenberg did not estimate the gastric acidity in her studies, it is interesting to learn that heavy fatty meals ultimately resulted in the disappearance of the alkaline tide in the urine. Watson took no note of the many factors which neutralize the HCl in the stomach, nor were his stomach investigations long in duration. The work of Watson, if repeated over several days, would probably give the same results. Rannenberg remarks on the necessary precaution to be taken in estimating the pH of the urine to prevent air bubbling through, as this will alter the results. She also emphasizes the importance of estimating the pH of the urine as soon as possible after it has deen voided. Hubbard and Mumford (1923) in studying cases of achlorhydria estimated the pH of the urine at various intervals, and they conclude that the secretion of HCl is of primary importance as a cause of the changes found in the urine, for following the ingestion of an appropriate meal in the cases of achlorhydria "no alkaline tide is observable."

Dodds (1923), in a paper on variations in some of the urinary constituents and the alveolar air CO_2 tension in relation to meals, remarks in his conclusions that the first meal of the day was followed by a decrease in the excretion of acid and ammonia calculated per hour; this alkaline tide lasted for one to two hours and was followed by a period of increased excretion of acid and ammonia per hour. The possible diurnal origin of these changes was excluded by starvation experiments.

The present investigation is a further attempt at correlating gastric secretion, especially in pathological conditions of the stomach, with the changes

in the reaction of the urine. The stomach juices were withdrawn at regular intervals over a period of 2–3 hours, during which period specimens of urine were also obtained and examined in half-hourly intervals. The procedure adopted was for the patient to be at rest, if possible in bed, without exercise, to empty his bladder on waking, then again immediately before the passage of the stomach-tube and every half hour following for a period of three hours. The pH of the urine samples was determined as rapidly as possible by means of the hydrogen electrode. The meal given to the patient was the standard gruel.

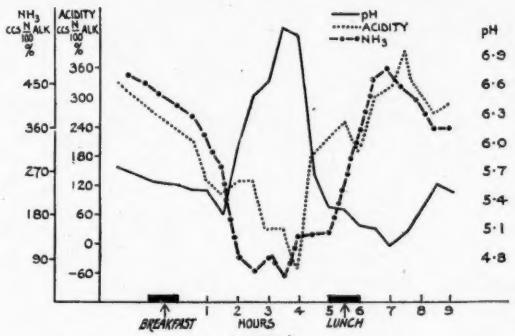

CHART 1.

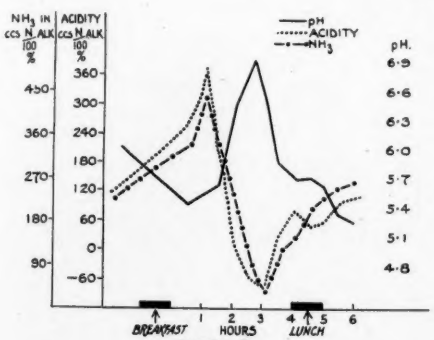

CHART 2.

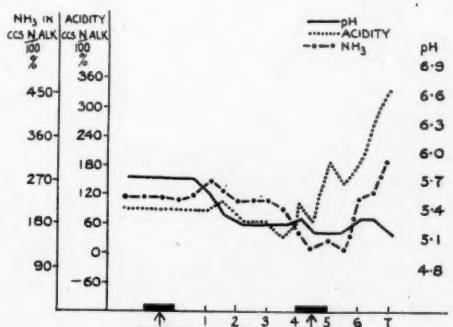

CHART 3.

In order to show the close parallelism of the urinary pH and the total acidity and ammonia excretion, three charts (Charts 1, 2, 3) are shown of observations extending over a period of six hours on three persons on a normal diet; the first two cases showed a normal secretion of HCl, and in the third one no HCl was demonstrable. The first two show a definite alkaline tide, and *pari passu* with a rise in pH the acidity and ammonia fall. The third example shows no alkaline tide, and there is practically no change until one and three-quarter hours after the meal, when the acidity rises. The acidity ammonia values and are given in c.c. N/100 NaOH % while the pH was estimated with the hydrogen electrode.

Eight cases of pernicious anæmia were investigated. The urinary findings were remarkably constant; with the exception of one case there was no greater variation than 0·3 pH for a period of three hours. The stomach secretion was investigated at the same time and complete achlorhydria was found in each case. Below is a typical chart of one of these cases showing the urine values superimposed on the fractional test-meal chart (Chart 4).

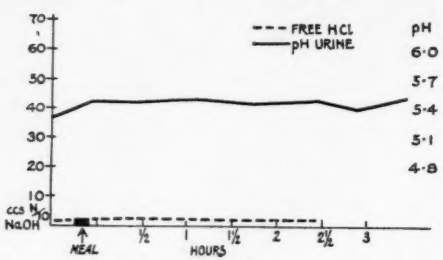

CHART 4.

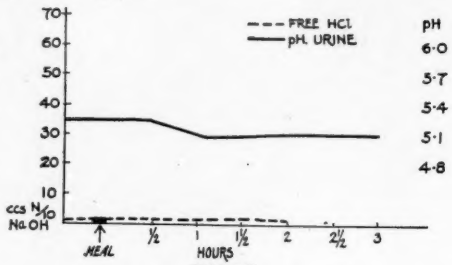

CHART 5.

A case of subacute combined degeneration of the cord was also investigated and the urinary findings were again constant. Below is a chart of this case showing no urinary change in pH (Chart 5).

In one case of pernicious anæmia, where there was marked regurgitation of bile, an acid tide was present in the urine. Whether this observation and the regurgitation of bile bear any relationship we cannot prove. The condition is charted below (Chart 6).

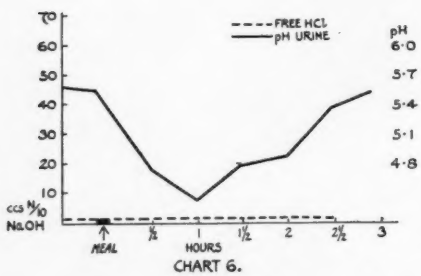

CHART 6.

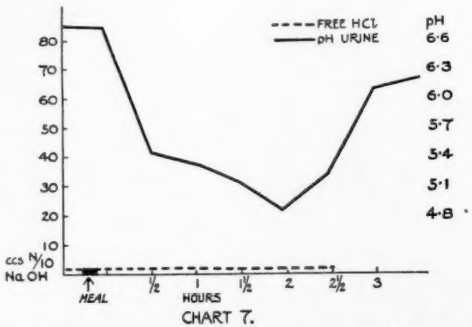

CHART 7.

It is interesting to recall that Bennett and Dodds (1921), when correlating the CO_2 alveolar air changes and gastric secretion in pernicious anæmia and other conditions of achlorhydria, found values which varied little, neither was there a marked rise or a marked fall in the CO_2 tension of the alveolar air following the morning meal. Normally the rise in tension follows the acid secretion of the stomach and the fall in tension follows the alkaline pancreatic secretion. From these findings they suggested that there might be some hyposecretion of the intestinal juices as well as achlorhydria in that condition. The urinary findings in the cases of pernicious anæmia examined here bear a striking resemblance in the complete absence (with the exception of one case)

of any significant change either way in the urinary pH. From the small number of these cases it would be inadvisable to draw any definite conclusion but the constancy of the urinary pH in this series is noteworthy.

The following are the variations in pH of the urines of the individual cases:

Case.	Diagnosis.	pH of urine. Initial pH.	½ hr.	1 hr.	1½ hrs.	2 hrs.	2½ hrs.	3 hrs.
J. H—	Pernicious anæmia	5·3	5·41	5·4	5·3	5·3	5·2	5·3
W. H—	,,	5·3	5·45	5·4	5·4	5·3	5·3	5·4
M. J—	,,	5·06	5·05	5·1	5·05	5·0	...	...
E. R—	,,	5·75	5·75	5·7	5·6	5·6	...	...
E. H—	,,	6·3	6·2	5·8	6·0	6·2	6·3	...
A. J—	,,	5·3	5·3	5·35	5·3	5·3	5·0	...
J. T—	,,	5·5	4·75	4·45	4·75	4·85	5·35	5·3
W. C—	Subacute combined degeneration	5·3	5·2	5·1	5·1	5·08	...	...

In marked contrast to these is a case who was in hospital following hæmatemesis. The diagnosis of the condition was cirrhosis of the liver. The test-meal showed complete achlorhydria. The stomach emptied rapidly, starch being no longer present after three-quarters of an hour. The urinary changes show a marked "acid tide"; in this case it is reasonable to assume that the rapid emptying means rapid stimulation of the pancreatic juices and in consequence this is rapidly reflected in the urine as an acid tide (Chart 7).

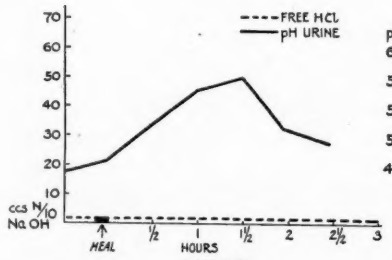

CHART 8.

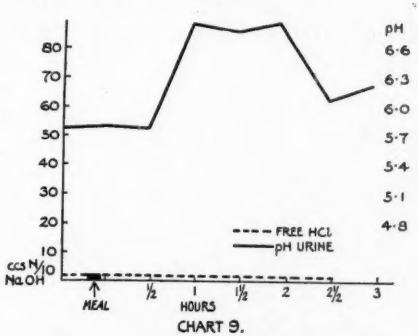

CHART 9.

Another case—carcinoma of the stomach—showed an apparent achlorhydria, but there was free regurgitation of bile which neutralized the free HCl that had presumably been secreted, for there was an alkaline tide (Chart 8).

The next case, an Indian student, showed no free HCl in his stomach juices, but the mucus was so abundant and tenacious that the test-tubes could almost be inverted without spilling the contents. The urinary changes showed a slight alkaline tide, possibly proving that acid was being secreted, but that it was not titratable owing to the mucus (Chart 9).

Three other cases deserve special mention in that they had an almost constant line of pH in the urine; these will be considered separately.

The first was a young person quite free from any digestive symptoms other than an occasional attack of vague dyspepsia which would be accompanied by

a slight icteric tint of the conjunctivæ. The fractional test-meal showed an almost complete absence of HCl except in the fourth specimen, when a value equal to 10 c.c. N/10 NaOH was found and the pH of the urine was shown to be almost constant. The chart is given below (Chart 10).

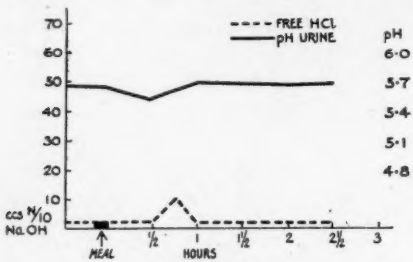

CHART 10.

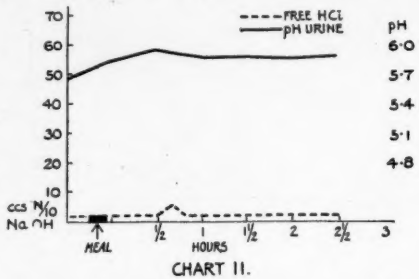

CHART 11.

The next case is that of a man who suffered from general carcinomatosis of the abdomen, in which his stomach and pancreas were grossly involved, but the pyloric exit was not obstructed. The fractional test-meal showed just a trace of HCl and there was no tide in the reaction of the urine. It is legitimate to assume that such a diffuse state of carcinoma will inhibit the normal secretion, and it is possible that in this case both gastric and pancreatic secretions were affected to an extreme degree and that this is reflected in the urine, as shown below (Chart 11).

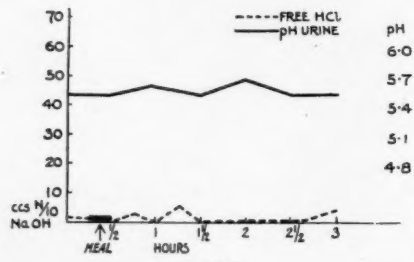

CHART 12.

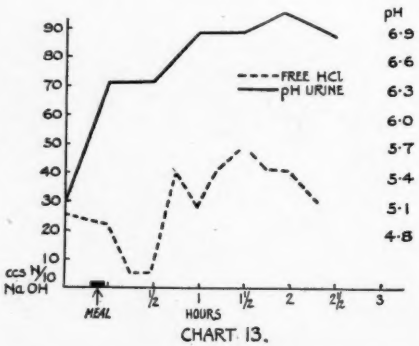

CHART 13.

A very similar condition is the third case—a primary carcinoma of the stomach which involved all the lesser curvature and had extended into the pancreas. The secretion of HCl was almost *nil* and the urinary reaction showed no change (Chart 12).

In two of the cases of pernicious anæmia histamine was injected during the repetition of the test-meal; no alteration in either the juices of the stomach or the pH of the urines was demonstrable.

The significance of this constancy in urinary acidity is that it gives an alternative method in the investigation of any case that presents an apparent

achlorhydria. There are many fallacies and sources of error, but if the estimations are done within a short period after voiding the urine, and especially if repetition of the test-meal reveals no change in the urine, or at least no alkaline tide, then it can reasonably be presumed that this is a case of absolute achlorhydria. Cases in which the absence of HCl is due to external neutralization, *i. e.* in the stomach itself, give an alkaline tide, and it is only in those in which there is no secretion of HCl that the pH of the urine does not rise. It is also possible that the effects of treatment could be observed. For example, an atonic stomach which before medical treatment showed absence of free HCl and no alkaline tide, would, after improvement, show a definite alkaline tide as the result of the morning meal. The return of HCl in the stomach would be accompanied by an alkalinity in the urine.

Cases in which HCl was present in the stomach were also investigated with regard to the urinary changes. The majority bear a certain resemblance and are only given in the form of figures; the more interesting cases are graphed on their fractional test-meal charts.

The first is a normal person showing a normal secretion of HCl with a definite alkaline tide in the urine (Chart 13).

The changes in a case of duodenal ulcer are shown below. The acidity reached in the stomach juices is high, and coinciding with this is the rapid alkaline change in the urine—a height reached almost at the outset and replaced as the digestion proceeds by a gradual fall in the pH. Whether this fall accompanies and is possibly due to rapid alkaline secretion in the duodenum is a point that is not yet proved (Chart 14).

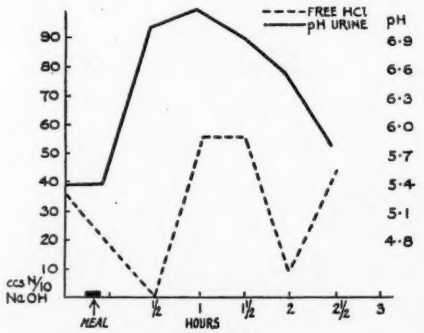

CHART 14.

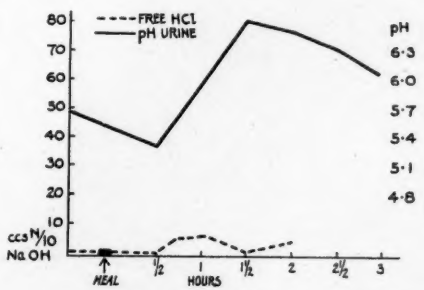

CHART 15.

Another person, a laboratory worker, shows free regurgitation of bile throughout his test-meal, with the result that the free HCl value is very low; notwithstanding this neutralization, a process which is really external to the acid-base equilibrium, there is a decided alkaline change in the urine (Chart 15).

One of the cases examined—a young laboratory worker—became very nervous while swallowing the stomach-tube for the first time; his fractional test-meal shows no free HCl until the third specimen. Coinciding with this

is the straight line of the pH of the urine over the corresponding period; this is followed later by a rise in alkalinity (Chart 16).

It is conceivable that as the reaction of the urine is affected by the acid poured into the stomach, it may also be affected by other digestive secretions. The next chart (17) is such an example. From a young person who salivated

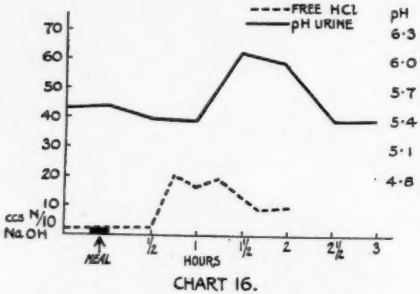

CHART 16.

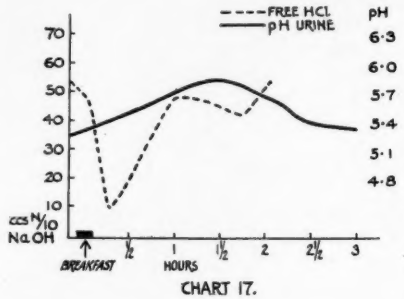

CHART 17.

profusely: 400 c.c. of saliva were collected in two and a quarter hours, and the resting juice of the stomach was 95 c.c. in volume. Here, although there is a definite secretion of HCl the alkaline tide is absent, and it is reasonable to assume that the loss of bases *viâ* the saliva during the period of digestion, which may have been enhanced by the tube-swallowing, counterbalances the loss of acid from the stomach; in consequence little alteration is found in the reaction of the urine. It was unfortunate that the case could not be repeated (Chart 17).

The following are the remaining cases which showed free HCl in their fractional specimens, with the various pH determinations every half hour:

Diagnosis.	pH of urine.						
	Initial pH.	½ hr.	1 hr.	1½ hrs.	2 hrs.	2½ hrs.	3 hrs.
Carc. ventriculi	6·3	6·3	6·8	7	7·2	6·9	6·6
Pyloric spasm	5·8	7·0	8·13	8·8	6·9	6·4	...
Duodenal ulcer	5·2	7·1	7·1	7·0	6·7	6·5	...
Normal person	5·4	5·35	7·2	7·6	6·4	5·2	5·0
"	5·3	5·5	5·7	5·9	5·7	5·3	5·3
Gastric ulcer	5·7	6·5	7·5	6·6	7·1	6·0	5·6
Normal person	5·5	5·4	5·5	6·1	5·8	5·3	5·3
Pyloric spasm	5·5	5·3	6·1	6·7	6·5	6·4	6·1
Gastric ulcer	5·3	5·9	6·2	6·4	6·6	6·5	6·4
Duodenal ulcer	5·4	7·1	7·5	7·0	6·5	5·7	...
"	5·7	6·7	6·6	7·3	7·4	6·8	6·2
Normal person	5·5	5·5	5·8	6·9	6·3	5·7	5·2
Duodenal ulcer	5·5	7·0	6·8	6·5	6·2	5·6	5·3
Visceroptosis	5·2	6·9	7·2	6·6	6·9	...	...
Colitis	6·1	6·5	6·9	7·4	8·1	7·0	...
Gastritis	5·3	6·4	6·4	7·0	7·1	7·2	...
"Hyperchlorhydria"	5·6	5·8	6·8	7·6	6·7	...	...
Duodenal ulcer	5·2	5·2	5·5	5·5	5·4	...	...
Pyloric ulcer	5·2	5·5	6·2	6·6	6·2	...	...

Conclusions.

In cases of true achlorhydria the reaction of the urine is remarkably constant, and it may afford an additional means of diagnosis in conditions where achlorhydria is the rule.

It also affords, for distinguishing between true and false achlorhydria, a method less fatiguing than the estimation of the total and mineral chlorides of the gastric secretion.

An acid tide may be obtained in cases in which there is rapid evacuation of stomach contents and early stimulation of alkaline pancreatic juices.

The writer wishes to acknowledge the kindness and help of Prof. Dodds. The writer also wishes gratefully to record his thanks to the Physicians of the Middlesex Hospital for permission to investigate the cases under their care.

REFERENCES.

ADLER, A.—(1919) *Zbl. inn. Med.*, **40**, 442.
BENCE-JONES.—(1845) *Phil. Trans. Roy. Soc.*, p. 335.
CAMPBELL, J.—(1920) *Biochem. J.*, **14**, 603.
DIXON-MANN, J.—(1905) 'The Chemistry and Pathology of the Urine.' London (Griffin.)
DODDS, E. C—(1923) *Brit. J. Exp. Path.*, **4**, 13.
Idem, AND BENNETT, I.—(1921) *Ibid.*, **2**, 58.
FISKE, C. H.—(1921) *J. Biol. Chem.*, **49**, 163.
HUBBARD, R. S., AND MUMFORD, S. A.—(1923) *J. Amer. Med. Ass.*, **80**, 304.
LEATHES, J.—(1919) *Brit. Med. J.*, **2**, 165.
QUINCKE.—(1884) *Z. klin. Med.*, **7**, 22.
RANNENBERG, E.—(1926) *Arch. ges. Physiol.*, **212**, 601.
WATSON, E. M.—(1923) *J. Physiol.*, **58**, xviii.

BLOOD PHOSPHORUS IN HEALTH AND DISEASE: IV.—THE PHOSPHORUS CONTENT OF THE BLOOD IN DIABETES MELLITUS.

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DURING the last twenty years evidence has been accumulating to suggest that the organic and inorganic compounds of phosphorus are intimately concerned in the normal metabolism of carbohydrate. After a meal the concentration of inorganic phosphate in the blood falls perceptibly and remains below the fasting level for several hours. A similar, more pronounced fall has been shown to follow the administration of insulin (Wigglesworth, Woodrow, Smith and Winter, 1923), accompanied by a parallel diminution in the excretion of phosphate in the urine (Bolliger and Hartman, 1925[1]). It is not unreasonable to connect these phenomena with carbohydrate utilization, and many workers, inspired by the discovery by Harden and Young (1906) of the hexosephosphates formed during the fermentation of glucose by yeast, have attempted to show that similar intermediate phosphate-sugar compounds play an essential part in animal carbohydrate metabolism. Attractive though this hypothesis appears, it must be admitted that the evidence in its favour is more circumstantial than direct. Some of the evidence has been derived from the study of diabetic subjects.

Bolliger and Hartman (1925[2]) found that in severe diabetes the alimentary fall in inorganic phosphate did not take place. They concluded, as a result of this and of other experiments, that insulin is as essential to the metabolism of phosphate as to the utilization of carbohydrate. Friedlander and Rosenthal (1926) injected alkaline sodium phosphate intravenously and noticed a fall in the glucose concentration in *diabetic* subjects, which lasted for several hours, whereas in normal control subjects no change occurred. They inferred from these observations that, as the respiratory quotient was unaltered, synthesis of inorganic phosphate + glucose to hexosephosphate had occurred somewhere in the tissues.

Is the phosphorus metabolism seriously upset in diabetes mellitus?

In the present investigation one aspect of this problem has been studied by applying the method described by Kay and Byrom (1927[1]) of determining the phosphorus partition of the blood. This method has been used in a series of cases of diabetes mellitus at various phases of the disease, both before and after treatment.

In all about forty cases of diabetes mellitus were studied, together with a few examples of non-diabetic hyperglycæmia, hæmochromatosis and acromegaly diabetes.

Experimental Results.

It was found that the cases fell into three main groups, namely:

(A) Cases of slight or moderate severity.
(B) Severe diabetes.
(C) Diabetic coma.

In Table I the average values for the various groups are collected for comparison with the normal figures. The results are expressed throughout as milligrammes of phosphorus per 100 c.c. of blood.

TABLE I.—*Average Results.*

Group.	Inorganic.	Ester P.	Hæmatocrit.	P. index.	Lipin P.
Normal subjects (33 cases)	3·0	22·7	43	53	12·0
Group A.—Mild Diabetes:					
Untreated (8 cases)	3·3	26·8	44	61	12·8
Treated (7 cases)	3·2	23·1	42	56	12·8
Group B.—Severe Diabetes:					
Untreated (9 cases)	2·9	19·2	43	45	13·9
Treated (8 cases)	3·2	22·7	41	54	14·3
Group C.—Diabetic Coma:					
Fatal. Untreated (3 cases)	7·6	12·7	47	27	16·3
Fatal. After insulin (1 case)	2·3	16·0	44	36	18·0
Not fatal. Untreated (1 case)	3·2	11·3	45	25	...
Not fatal. After insulin (4 cases)	2·6	23·0	43	52	13·8

N.B.—Owing to the high cost of printing, complete analytical protocols cannot be given here.

Analysis of Results.

Group A.—Cases of Mild Diabetes.

The members of this group all show some degree of impairment of carbohydrate tolerance but little or no ketosis. The majority of the patients are elderly and the essential abnormality would appear to be deficient storage of carbohydrate.

In the untreated cases the only change is a slight increase in the ester phosphorus content of the corpuscles, as indicated by the raised phosphorus index.

The effect of treatment is to restore the ester phosphorus and the phosphorus index to normal.

Group B.—Cases of Severe Diabetes.

This group consists of more severe cases of diabetes with marked ketosis. The patients, on the whole, are young adults in whom, in addition to impairment of storage power, deficient ability to burn up sugar, with secondary changes in fat metabolism, are present.

In the untreated cases there is a more marked disturbance of the phosphorus balance in the blood. While the inorganic phosphate remains within normal limits the ester phosphorus is consistently below the normal level, the phosphorus index being below 50 in every case (average index for normal individuals = 53). In those cases examined after satisfactory treatment by dietetic restriction and insulin, the ester phosphorus and the phosphorus index are, with a single exception, normal. In three cases the opportunity was taken of performing analysis before and after treatment on the same patient. In each case (Table II) the readings confirm the above findings.

TABLE II.

Case.	Inorganic P.	Ester P.	Hæmato-crit.	P. Index.	Lipin P.
(1) Before treatment	3·2	17·4	42	41	14·6
After ,,	3·4	22·1	44	55	16·0
(2) Before ,,	3·1	20·6	45	46	13·8
After ,,	3·5	20·5	37	55	16·0
(3) Before ,,	3·3	16·9	38	44	13·8
After ,,	3·5	24·7	39	63	14·0

Group C.—Cases of Diabetic Coma.

Since the introduction of insulin, untreated cases of diabetic coma are rarely met with in hospital. The values obtained, however, in the few cases available are so strikingly and consistently abnormal that they may be accepted as typical of the condition. In untreated diabetic coma the diminution in the ester phosphorus is extreme, the fraction which resists phosphatase hydrolysis being chiefly depleted. In addition there is a very substantial rise in the inorganic phosphate in those patients who die in coma. In one case the inorganic phosphate was as high as 11·4 mgm. % (normal = 3·0 mgm. %).

The effect of treatment by insulin, if successful, is very striking: the ester phosphorus rapidly rises to the normal level.

The following readings, obtained from a case of diabetic coma, are sufficient illustration of the extent of the change.

	Inorganic P.	Ester P.	Hæmatocrit.	P. index.
Before insulin	3·2	11·3	45	25
Five days after commencing insulin	3·3	25·0	41	60

General Discussion.

The inorganic phosphate.

In diabetes there is little or no change in the inorganic phosphate content of the blood (excepting the temporary fall after insulin injection) until coma supervenes, when the level rises very considerably. It is not easy to account for this change. It may be suggested that as the phosphate falls in hypoglycæmia, the opposite condition of hyperglycæmia should be accompanied by a rise in the phosphate level. No such parallelism however is found to occur. In the normal individual the phosphate falls after a meal, while the blood sugar rises. In diabetes, many cases are recorded in the present paper with marked hyperglycæmia, yet without alteration in the phosphate level. In one case of non-diabetic hyperglycæmia, caused by hæmorrhage into the fourth ventricle, the phosphorus content of the blood was, again, strictly within normal limits. A second explanation is that the rise in phosphate is the result of acidosis. Kay (1924) observed a slight increase in the inorganic phosphate in ammonium chloride acidosis, but it is improbable that acidosis alone can be held responsible for the very high values here recorded.

A more probable suggestion is that the increase is brought about by retention of phosphate by damaged kidneys. It has been established (De Wesselow, 1923; Byrom and Kay, 1927[2]) that in the uræmia which accompanies severe renal destruction the phosphate content of the blood rises to very high levels. Furthermore, Fitz (1917) and other workers have found evidence of considerable renal damage in severe diabetes. It seems likely, therefore, that the high phosphate level in diabetic coma is the result of renal damage. There is no doubt that a proportion of cases of diabetic coma, especially if treatment is delayed, do not respond to insulin, even in the absence of sepsis. The fact that a high phosphate value has been recorded in each of the three fatal cases of the present series lends colour to the view that in such cases renal damage is to a large extent responsible for the fatal termination.

The ester phosphorus.

The fall in the ester phosphorus content of the corpuscles is the most striking result of the investigation. In diabetic coma the corpuscle contains less than one half of the normal amount, while in severe uncomplicated diabetes there is a similar, but less extreme deficit. *Effective insulin treatment restores the ester content of the corpuscles to normal.*

Such a depletion of ester phosphorus has not been found in any other disease studied in this laboratory. In a series of about 150 analyses of blood obtained from various pathological and normal subjects, in no instance has an index of below 48 been encountered, yet out of eleven examples of severe untreated diabetes only two show an index above this value, while in coma the index readings fall as low as 28 and 24.

In searching for an explanation the following possibilities have been considered.

(1) In the polyuria of diabetes it is possible that an excessive amount of

inorganic phosphate is excreted by the kidneys, the ester compounds of the corpuscles then being hydrolysed to meet the deficit. In diabetic coma, however, polyuria is often superseded by scanty secretion of urine or even anuria. Furthermore, with the polyuria of chronic nephritis (Byrom and Kay, 1927[2]) and diabetes insipidus the ester phosphorus of the corpuscles is actually raised.

(2) Insulin may be essential to the synthesis of the ester compounds of the corpuscle from the inorganic phosphate of the plasma. As the severity of disease increased, the paucity of available insulin would lead to a fall in the ester phosphorus of the corpuscle and an accumulation of inorganic phosphate in the plasma. Such an accumulation would be rapidly relieved by the kidneys (for it is found that injected phosphates are rapidly excreted), unless these organs were badly damaged, as they are in diabetic coma.

(3) Acidosis may be responsible. Kay (1924) investigated the phosphorus partition in severe acidosis induced in a normal subject by ingestion of ammonium chloride, and recorded a fall in the ester phosphorus content of the corpuscles. The phosphorus index (calculated from Kay's data) fell from the normal level of 53 to a minimum of 39, remaining subnormal for several days.

Martland (1925) has found that diminution of the pH of the blood tends to increase the hydrolysis of the ester phosphorus, while an increase of the pH favours synthesis.

In the present series of cases the fall in the ester phosphorus is greatest in those cases in which acidosis, as judged by gross ketonuria and symptoms of coma, is most marked, while in mild diabetes, without ketosis, there is no such change. It may be assumed that the low ester phosphorus content of the corpuscle in severe untreated diabetes is probably the direct result of acidosis.

Changes in the hæmatocrit.

In diabetic coma the hæmatocrit tends to rise. As Kay observed the same phenomenon in ammonium chloride acidosis it would appear that acidosis is in some way responsible. Insulin tends to counteract this increase.

The lipin phosphorus.

The lipin phoshorus was determined, on whole blood in all cases, and on plasma in 14 cases. The results are in complete accordance with the findings of Bloor (1916). There is an increase in the plasma lipin phosphorus in many cases of severe diabetes, the amount of lipin in the corpuscles remaining, as in nephritis, relatively normal. At the same time fatal coma may occur in the absence of any marked increase is the blood lipin, while on the other hand high readings are occasionally met with in quite mild cases of diabetes. Bloor remarked on the rarity of naked-eye lipæmia in diabetes; in the present series of about 40 cases no instance of milkiness of the plasma was met with, even when the lipin exceeded double the normal value. The lipin phosphorus in diabetes differs in its reaction to treatment. Whereas the inorganic and

ester phosphorus are restored to normal after treatment the lipin frequently remains unchanged or is even increased. This is possibly due to the fact that the average diabetic diet usually includes appreciably more fat than that of the normal individual or the untreated diabetic.

Conclusions.

(1) In severe untreated diabetes and in diabetic coma there is a pronounced fall in the organic acid-soluble phosphorus ("ester phosphorus") of the blood-corpuscles, which appears to be the result of acidosis.

(2) After satisfactory treatment by dietetic restriction and insulin, the ester phosphorus returns to the normal level.

(3) In mild diabetics and in cases of moderate intensity the only abnormality is a small increase in the ester phosphorus content of the corpuscles.

(4) In fatal diabetic coma the inorganic phosphate rises very considerably, probably as the result of renal damage.

(5) The conclusions of Bloor with regard to the lipin content of the blood in this disease are confirmed.

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REFERENCES.

Bloor, W. R.—(1916) *J. Biol. Chem.*, **26**, 424.
Bolliger, A., and Hartman, F. W.—(1925[1]) *Ibid.*, **64**, 91.—(1925[2]) *J. Amer. Med. Ass.*, **85**, 653.
Friedlander, K., and Rosenthal, W. G.—(1926) *Arch. exp. Path. Pharmak.*, **112**, 65.
Fitz, R.—(1917) *Arch. Int. Med.*, **20**, 809.
Harden, A., and Young, W. J.—(1906) *Proc. Chem. Soc. Lond.*, **21**, 189.
Kay, H. D.—(1924) *Biochem. J.*, **18**, 1123.
Kay, H. D., and Byrom, F. B.—(1927[1]) *Brit. J. Exp. Path.*, **8**, 240.—(1927[2]) *Ibid.*, **8**, 429.
Martland, M.—(1925) *Biochem. J.*, **19**, 117.
de Wesselow, O. L. V.—(1923) *Lancet*, **205**, 163.
Wigglesworth, V. B., Woodrow, C. E., Smith, W., and Winter, L. B.—(1923) *J. Physiol.*, **57**, 447.

FOWL-POX ANTISERUM.

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IN a former article (1925) we wrote as follows: "Serum from immunized hens which received several inoculations of dead, and, later, living, infective material has been used experimentally for protective purposes. We have done but a few experiments up to now but in two completed experiments the serum of immunized hens when injected into normal birds has protected them against a subsequent inoculation of virulent material."

Since our first article was published, Findlay (1928) has shown that "hyper-immunization with fowl-pox virus produces in the fowl an antiserum containing viricidal antibodies," and we ourselves have carried out many experiments which show that fowls can produce serum possessing viricidal antibodies for the fowl-pox virus. All our work has been done on fowls artificially infected with the virus; we have not had the opportunity to test our hyper-immune serum on birds suffering from a natural infection.

PRODUCTION OF ANTISERUM.

Our early method was to inject normal fowls intramuscularly with increasing doses of killed virus, in the form of ground-up heated comb-scales suspended in saline, followed by several injections of living virus. Lately we have made use of fowls which have already been immunized by comb-scarification. Such birds are used in the production of fowl-pox vaccine, the procedure being to scarify the comb and apply some virulent comb-scales from an infected fowl. When a crop of scales has been produced it is removed and further scarification is carried out. It is soon found that the bird is incapable of further production of scales, *i.e.* it has been immunized and will no longer "take" when further scarified. Living virus in now inoculated intramuscularly and after four to six increasing doses a hyper-immune serum is produced.

THE TESTING OF SERUM.

In our testing of the viricidal properties of such hyper-immune serum we believe it is important to determine as closely as possible the minimum infecting dose of our test-virus. It is probable that some workers in the past have failed to demonstrate antibodies in serum because they used an overwhelming dose of virus when testing the serum.

The Test Material.

The test material used consists of comb-scales from an infected bird scraped off about the tenth to twelfth day after infection. The scales are dried for a few hours at 37°C. and are then powdered finely in a mortar. To

produce a very fine powder, the mortar-ground material is further ground up in a ball mill for about two hours; this results in the production of a fine grey powder rich in fowl-pox virus. Experiments have shown that prolonged grinding will reduce the infective properties of the powder and may eventually destroy any virus present. It is of interest that similar grinding renders *B. tuberculosis* non-infective. The powder is now stored *in vacuo* and has been shown to retain its infective properties with no demonstrable loss over a period of at least twelve months. For testing serum, 0·1 c.c. of a selected powder is suspended in 5 c.c. saline and after thorough mixing in a mortar is filtered through cotton-wool. A dilution of 1/10 of this material contains about fifty infecting doses and is used by us as a test-dose. We thought at one time that it would be possible to use a Kieselguhr filtrate of such a powder for testing purposes. This is of low value (*vide* Table I) and not suitable for use in the test.

TABLE I.—*Infecting Value of Fowl-Pox Virus Before and After Filtration.*

Sample.	Dilution.	Reactions. Before filtration.	After filtration.
A.	1/100	+ + +	+ + +
	1/1000	+ + +	+ +
	1/10,000	+ +	+
	1/100,000	+	−
	1/1,000,000	±	−
B.	1/10	+ + +	+
	1/100	+ +	−
	1/1000	+	−
	1/10,000	±	−

+ + +, + +, +, ± = degrees of reaction on fowl's comb. − = no reaction.

The Actual Tests.

Hyper-immune fowl-pox antiserum can be tested in two ways by (*a*) the scarification method and (*b*) the intravenous injection method. Our experiments show that a given dilution of emulsion of powder which causes lesions when applied to a scarified area of a fowl's comb will, in a dose of 1 c.c. injected intravenously, produce generalized lesions on eye, comb and throat in a normal fowl.

(*a*) In testing serum by the scarification method a measured amount of the test-dilution of virus is mixed with the same volume of diluted serum. After standing at room temperature for an hour the mixtures are rubbed into scarified areas of a normal fowl's comb. It is possible to test at least twelve samples on the comb of one bird. Normal fowl-serum and saline solution are used as controls on the comb of every bird. Table II is an example of the results of the testing of one sample of serum.

(*b*) To test serum by the intravenous injection method several fowls, preferably young cockerels, must be used. Varying amounts of serum are injected intramuscularly into these birds and on the following day each bird receives 1 c.c. of the test-dilution of the fowl-pox virus intravenously. The

birds are kept under observation for about 14 days; lesions seldom develop later. Table III is an example of the testing of a sample of serum by this method. We have made many sera similar to those shown in Tables II and III.

TABLE II.—*Testing Fowl-Pox Antiserum by the Scarification Method.*

Fixed test-dilution of virus containing about 50 infecting doses mixed with equal quantity of serum and rubbed into scarified area on fowl's comb.

Dilution of serum to be tested.	Reaction.
1/2	−
1/4	−
1/8	−
1/16	−
1/32	−
1/64	±
1/128	+
1/256	+ +
1/2 (normal fowl-serum)	+ +
No serum	+ +

TABLE III.—*Testing Fowl-Pox Antitoxin by the Intravenous Method.*

Varying doses of serum injected intramuscularly 24 hours before 1 c.c. test-dilution of fowl-pox virus (containing about 50 infecting doses) injected intravenously.

Fowl.	Amount of serum.	Reaction.
1	1 c.c.	−
2	0·5 c.c.	−
3	0·25 c.c.	±
4	0·1 c c.	±
5	0·05 c.c.	+
6	0·025 c.c.	+ +
7	1 c.c. (normal fowl serum)	+ +
8	No serum	+ +

+ +, +, ± = degrees of reaction. − = no reaction.

Although up to the present we have been unable to test the value of hyper-immune serum in the field, we believe that it would prevent infection in a naturally occurring outbreak, for in all probability the infecting dose of virus under natural conditions is much less than that used in our experiments.

SUMMARY AND CONCLUSIONS.

Fowls can be so immunized with injections of living virus against fowl-pox that their serum shows marked viricidal properties. The degree of protective value of the serum may be estimated by the two methods described —scarification and injection.

REFERENCES.

DALLING, T., ALLEN, H. R., AND MASON, J. H.—(1925) *Vet. Rec.*, **5**, 561.
FINDLAY, G. M.—(1928) *Proc. Roy. Soc.*, **102B**, 354.

THE GLYCOLYTIC ACTION OF SOME TUMOURS AND THE EFFECT OF INSULIN.

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THE work of Warburg (1926, 1927[1,2]) and his collaborators has demonstrated a pronounced quantitative difference between the metabolism of normal and cancerous tissue. As a result of a study of the behaviour of the tissue towards carbohydrate under aerobic and anaerobic conditions, the following broad generalizations have been arrived at:

(1) Under aerobic conditions normal tissues derive their energy almost entirely from oxidation of carbohydrate (respiration). Under anaerobic conditions, however, they can to some extent (usually small) split carbohydrate to lactic acid (glycolysis).

(2) Under aerobic conditions tumour tissue not only brings about oxidation of carbohydrate in the usual way, but also exhibits aerobic glycolysis, producing large amounts of lactic acid. Under anaerobic conditions the extent of glycolysis is also very great.

These results have been made the basis of several systems of classification in which attempts have been made to differentiate normal and malignant tissues on the basis of their metabolism. Some exceptions to the above statements are known and the whole problem is one of considerable complexity. In Warburg's publications the use of an exceedingly delicate manometric method is described, for the measurement of the respiration and glycolysis of a few milligrammes of surviving tissue, and it is mainly with the aid of this technique that the results mentioned above have been attained.

Warburg clearly states that the manometric method is non-specific; that is, the exact chemical nature of the products of the reactions must be established by analysis before the method can be applied to a given problem. Thus the formation of any acid by the tissue would cause the liberation of carbon dioxide from the bicarbonate and a corresponding increase of pressure would be indicated by the manometers. Warburg has, therefore, applied specific methods of chemical analysis to the problem and in one experiment (Warburg, 1925), using Jensen's rat sarcoma in Ringer-glucose solution

containing nitrogen, he has demonstrated that the acid formed is in fact lactic acid. This is established by its isolation in the form of the zinc salt, the satisfactory chemical identification of which is described. The yield of lactic acid obtained in this experiment by calculation from the weight of its zinc salt was less than that expected from the manometric measurements by some 50 %. Warburg accounts for this apparent discrepancy on the ground that the sections of tissue used in an experiment such as this, in which several grammes are required, are not so thin, and cannot be so carefully selected as in an experiment in the manometer vessel, which requires only a single section. Moreover, the shaking and mixing with the fluid are also less efficient.

In a few cases Warburg has determined by the usual analytical methods the amount of lactic acid formed. Thus the manometric method is compared with the chemical one in one or two early experiments (Minami, 1923; Warburg, Posener and Negelein, 1924), using the Fürth-Charnass method for estimating the lactic acid, and in a later experiment (Stahl and Warburg, 1926) the method of Clausen (1922) is employed. More recently the lactic acid produced by a tissue-culture *in vitro* has been determined by the same method (Wind, 1926). These results are in satisfactory agreement with the manometric determinations, but the number of experiments is not very large, and it appeared that further determinations might be of value.

In the present paper we describe experiments on the anaerobic glycolysis of some tumours. Determinations of lactic acid have been made by Clausen's method, using the modification of Friedmann, Cotonio and Shaffer (1927). A number of controls have shown the method to be trustworthy; 33 estimations on a solution of pure zinc lactate gave a mean recovery value of 99·4 %, the extremes being 104 % and 94 %. Glucose estimations have been carried out by the method of Folin and Wu. Although these methods are capable of detecting changes of a few milligrammes per cent., they are not, of course, sufficiently sensitive for reliable use in experiments in which only a few milligrammes of tissue are employed. We have, therefore, been compelled to use larger quantities of tissue; in most experiments about 100 mgm. dry weight. This led to difficulties similar to those encountered by Warburg in the experiment described above on the isolation of zinc lactate. In consequence our results are quantitatively in agreement with that experiment rather than with the manometric observations.

In the experiments to be described, sections as thin as practicable were cut from the fresh tissue with a razor; these were placed in Ringer solution containing glucose (0·2 %) and sodium bicarbonate (0·21 %), 2·5 gm. of moist tissue being used per 100 c.c. of Ringer solution in most cases. In some experiments the tissue was minced in a small machine such as is used for tumour implantations. In all experiments the atmosphere was nitrogen containing 5 % by volume of carbon dioxide. The fragments of tissue were dispersed in a meshwork of glass-wool in a test-tube containing the Ringer solution; this was kept in a water-bath at 38° C., and the gas mixture from a cylinder was bubbled continuously through the fluid. This arrangement served to maintain a constant circulation of fluid round the tissue fragments and at the same time kept the pH constant at about 7·4.

The removal of interfering substances prior to the oxidation of the lactic

acid has been thoroughly described in the two papers mentioned. In the present case the main impurity was glucose, the amount of proteins present in solution being small. Hence Van Slyke's alkaline copper reagent was found to be adequate, and this also removes the small amount of protein. For this purpose 5 c.c. of solution (filtered if necessary) were removed from the reaction tube, placed in a 50 c.c. volumetric flask, 10 c.c. distilled water and 10 c.c. of 20% copper sulphate solution were added. After mixing, 10 c.c. of 10% $Ca(OH)_2$ suspension were added and, after making up to the mark with distilled water, the contents were allowed to stand for 45 minutes with occasional agitation. After filtering, 20 c.c. of the clear liquid were used for the estimation. In two cases, precipitation with tungstic acid (Folin and Wu) was used previous to the treatment with alkaline copper, and the results were found to be unaffected. In a later experiment we compared the above methods with one using ether extraction of the lactic acid. The figures are in satisfactory agreement.

In the ether extraction, 2 c.c. liquid, after acidification with sulphuric acid, were dehydrated with plaster-of-Paris and extracted for two hours in a Soxhlet apparatus with ether. This method we have also found useful for estimation of lactic acid in gastric analyses. To the ether was added 10 c.c. of distilled water, 1 drop of phenolphthalein and enough N/10 KOH solution to give a pink colour. The ether was distilled off and the solution evaporated to dryness on a water-bath. The residue was washed with hot distilled water into a 50 c.c. volumetric flask and portions of 25 c.c. were taken for the estimation. Blank values on the plaster-of-Paris and ether gave figures of 0·4 to 0·5 c.c. N/500 iodine, similar to those obtained with the copper sulphate reagent alone.

It has been suggested (Blanchetière, 1927) that the lactic acid production in certain of Warburg's experiments may be due to the presence of lactic acid producing organisms introduced with the fluid, either by contamination during manipulation or by the use of contaminated material such as tumour-tissue which has become infected in the animal. Such a criticism would certainly not explain the constant difference between tumour-tissues and normal tissues except by the assumption that all the tumours were infected. In actual fact, if non-ulcerated tumours are removed from animals while health is still good, perfectly sterile samples are, as a rule, obtained. There is, however, another consideration which completely excludes the possibility of bacterial origin of the lactic acid produced in Warburg's experiments, and that is the fact that even considerable contamination with lactic acid-forming organisms will not result in the formation of a significant amount of lactic acid in two hours under the conditions of the experiments. An experiment demonstrating this point was carried out as follows:

B. coli to the extent of 5 million organisms per c.c. was added to a minced tumour (Jensen's rat sarcoma) in glucose Ringer solution; a control without *B. coli* was made at the same time. In the control tube 4·84% of lactic acid was produced and in the contaminated tube 5·18% (*vide* Table B, experiments 11 and 12). This slight increase (0·34%) of lactic acid is not greater than the variation encountered in results obtained with different samples of the same tumour, and could not account for the wide differences between normal tissues and malignant growths.

In a paper by Cramer, Dickens and Dodds (1926), it was shown for a number of malignant tumours that these tissues do not contain insulin, or that they contain only a small fraction of the amount of insulin found in other normal body-tissues such as the spleen, liver and kidney. Among the tumours studied were Jensen's rat sarcoma, some mouse sarcoma and three kinds of human malignant tumours. It was suggested in that paper that the absence of insulin from these tissues might be connected with their abnormal carbohydrate metabolism, supposing the oxidation of glucose in the cell to be associated with the presence of insulin at the site of oxidation. There was, however, no direct evidence on this point available.

In view of this we have performed a series of experiments in which insulin was added to the Ringer-glucose solution used for glycolysis. The details of these experiments were otherwise as described above. It may be stated at once that we have observed no change in the rate of glycolysis due to the addition of insulin under the conditions described, and moreover, the amount of glucose consumed again corresponds closely with the lactic acid formed. These results apply to approximately anaerobic conditions.

Since thyroxin exerts its action by a general stimulation of metabolism, we have, for the sake of comparison, included a few experiments in which this substance was added to the Ringer-glucose solution used for glycolysis. No change was observed in the rate of glycolysis nor in the rate of consumption of glucose. In some experiments at present in hand, it is proposed to extend these results to aerobic conditions. Since this necessitates the measurement of respiration as well as of glycolysis, the manometric technique is being used for this purpose.

Experimental Results.

Calculation from control experiments showed that each 100 mgm. of tumour, prepared as described above and filtered off shortly after its addition to Ringer-glucose solution, yielded 0·4 mgm. of lactic acid, taking the average of a number of determinations. This correction is incorporated in the results tabulated below. A similar result was obtained by performing control experiments in which the Ringer solution containing tumour-tissue was cooled to 0° C., the conditions being otherwise as in a normal experiment. There is, therefore, no appreciable formation of lactic acid at this temperature, and the possibility of the extraction of appreciable quantities of preformed lactic acid from the tumour-tissue is also excluded.

The determination of the dry weight of the tissue presents considerable difficulty. In the majority of experiments the dry weight was checked by weighing further quantities of moist tissue, equal in weight to that taken in the main experiment, and drying for two hours at 110° C. This indirect method is unavoidable since it is very difficult to recover the whole of the tissue at the end of the experiment. These estimations gave an average dry weight equal to 18% of the wet weight, and the extreme figures were 16% and 20%. This method does not, therefore, introduce a very large error provided the draining of tissues before weighing is carried out similarly in all cases.

JENSEN'S RAT SARCOMA.

A. *Sections Cut with the Razor.* (Temperature 38° C.)

Experiment.	Dry weight of material per 100 c.c. Ringer (mgm.).	Time of experiment (hours).	Glucose consumed per 100 c.c. (mgm.).	Lactic acid formed per 100 c.c. (mgm.).	Lactic acid formed per hour per 100 mgm. tissue (mgm.).
1.	420	1	...	16·7	4·0
2.	450	1·5	44	40·0	5·90
3.	280	2	..	29·8	5·32
4.	450	2	49	39·9	4·43
5.	450	2	50	47·1	5·25
6.	450	2	59	54·8	6·09
7.	450	2	...	44·8	4·97
					Mean (excepting No. 1), 5·33%.

Note.—pH in No. 7 before experiment, 7·54; after experiment, 7·39 (glass electrode).

B. *Minced Tissue.* (Temperature 38° C.)

8.	900	2·3	...	78·7	3·8
9.	400	2·75	33	43·3	3·93
10.	450	2·5	...	54·7	4·86
11.	450	1·5	28	32·7	4·84
12. As No. 11, but with *B. coli* (gross contamination)	450	1·5	32	35·0	5·18
Ringer solution and tissue cooled in ice:					
13.	400	2	...	(1·1)	(0·15)
14.	900	2·3	...	(6·6)	(0·3)

C. *Hormones and Minced Tumour.* (Temperature 38° C.)

15. Normal control	900	2·3	...	78·7	3·8
16. With insulin (6 units per c.c.)	900	2·3	107	96·2	4·03
17. With thyroxin (0·1 mgm. per c.c.)	900	2·3	...	85·3	4·12
18. With adrenalin (0·2 „ „)	900	2·3	...	87·2	4·20

D. *Comparison of Glucose Consumption and Lactic Acid Formation with and without Hormones.* (Minced tumour; temperature 38° C.)

19. Normal control	400	2·75	33	43·3	3·93
	...	4·75	93	84·3	*
19*a*. Normal control	450	2	52	47·7	5·3
20. Ditto, with insulin (6 units per c.c.)	400	2·75	40	42·7	3·89
	...	4·75	97	77·3	*
20*a*. Ditto, with insulin (7 units per c.c.)	450	2	54	42·9	4·8
21. Ditto, with thyroxin (0·05 mgm. per c.c.)	400	2·5	35	33·3	3·33
	...	4·5	87	81·3	*

* Not calculated owing to volume being much reduced by lactic acid determinations.

E. *Berkefeld Filtrate from Jensen's Rat Sarcoma.* (Incubated at 38° C.)

22.	1500 (before filtration)	2	6	...	No change.

F. *Effect of Deprivation of Glucose before Experiment.*

Experiment.	Dry weight of material per 100 c.c. Ringer (mgm.).	Time of experiment (hours).	Glucos consumed per 100 c.c. (mgm.).	Lactic acid formed per 100 c.c. (mgm.).	Lactic acid formed per hour per 100 mgm. tissue (mgm.).
23. Sections kept 3 hours without added liquid before experiment	450	2	10	24·0	2·67
23*a*. Sections minced and kept 4 hours	450	2	49	30·2	3·35
24. Minced tumour kept in Ringer without glucose for 3½ hours before experiment	400	2	(21)	14·4	1·9
24*a*. Ditto	450	2	(47)	13·2	1·46
25. Minced tumour kept in Ringer with 0·2% glucose for 2 hours before experiment	400	2	21	31·4	3·92
25*a*. Ditto	450	2	61	49·7	5·50
26. As No. 24, then transferred to Ringer with glucose for 1 hour	450	1	52*	...	...
before experiment	...	2	49	25·2	2·8

* Glucose fell from 0·247 to 0·195% in 1 hour at room temperature and was then exchanged for fresh glucose Ringer (0·246% glucose).

G. *Rous Chicken Sarcoma.* (Temperature 38° C.)

(*a*) Fairly thick sections	450	2	36	36·7	4·0
(*b*) *Thick sections with some blood present	450	2	26	23·0	2·55
(*c*) *Ditto, with insulin (7 units per c.c.)	450	2	..	16·6	1·84

* In these experiments there was considerable delay in preparing sections.

H. *Some Normal Tissues.*

Specimen.	Dry weight of material per 100 c.c. Ringer (mgm.).	Time of experiment (hours).	Glucose consumed per 100 c.c. (mgm.).*	Lactic acid (mgm.). Per 100 c.c.	Lactic acid (mgm.). Formed.*	Lactic acid formed per hour per 100 mgm. tissue (mgm.).*
Rat kidney sections—						
at 37° C.	340	2	...	7·3	1·9	0·3
at 0° C.	360	2	...	5·4		
Rat liver minced—						
at 37° C.	260	2	...	3·7	...	...
Rat liver sections—						
at 37° C.	600	2	...	7·1	1·6	0·15
at 0° C.	400	2	...	3·7		
Rat heart-muscle sections—						
at 37° C.	450	2	10	6·3	4·0	0·45
at 0° C.	450	2	...	2·3		
Rat testes minced—						
at 38° C.	210	2	19	7·5	...	...
Ditto, with insulin (1 unit per c.c.)	210	1	7	...	...	...
	...	2	13	6·5	...	..

* Owing to the small differences from the blank values, the figures in these columns are approximate only.

Comparison of Manometric and Chemical Methods.

A direct comparison between the best manometric measurements and those obtained by analysis is not possible for the reasons already mentioned. In the following experiment considerable care was taken to obtain thin sections, and the manometric and chemical determinations were conducted simultaneously, two of the sections being used for the manometric determinations. The tumour-tissue was Jensen's rat sarcoma. The main experiment was in an atmosphere of nitrogen containing 5% carbon dioxide, but an experiment with oxygen containing 5% carbon dioxide is also included in the table below.

JENSEN'S RAT SARCOMA.

Experiment number.	Medium.	Atmosphere.	Lactic acid formed per 100 c.c.		Lactic acid % of dry weight per hour.	
			Usual method (mgm.).	Ether extraction method (mgm.).	Usual method (mgm.).	Ether extraction method (mgm.).
1.	Ringer without glucose	$N_2 + 5\% CO_2$	14·5	11	1·6	1·2
2.	Ringer with 0·2% glucose	$N_2 + 5\% CO_2$	80	77	8·8	8·55
3.	Ringer with 0·2% glucose	$O_2 + 5\% CO_2$	36	40	4·0	4·4

Time of experiment 2 hours; temperature 38° C.; dry weight of tumour in each tube 45 mgm. in 10 c.c. Ringer.

As a result of the manometric measurements under similar conditions (38° C., in Ringer with 0·2% glucose; N_2 + 5% CO_2) we obtained $Q_M^{N_2}$ = (a) +28·9; (b) +28·2; mean +28·6. This result (expressed above in Warburg's nomenclature as c.mm. CO_2 liberated per mgm. tissue per hour) when multiplied by the factor 0·4 gives the equivalent lactic acid, calculated as percentage of the dry weight; in this case 11·6%. This figure compares reasonably well with the results (8·8%, 8·5%) obtained by analysis, if the difficulties of the larger scale experiment are taken into account. The sections chosen for the manometric experiment were slightly thinner than the majority of those used in the other case, and this would also tend to emphasize the difference between the results. Warburg, by the isolation of zinc lactate, obtained lactic acid equivalent to 7·7% of the dry weight of Jensen's rat sarcoma per hour.

Conclusions.

(1) In agreement with the work of Warburg, our experiments have shown a marked difference between certain normal tissues and malignant tumours as regards the anaerobic metabolism of glucose.

(2) Using a simple technique and a direct chemical method of estimating lactic acid, the tumour-tissues were found to produce about 5 to 10 times as much lactic acid as was produced by normal tissues under the same conditions.

(3) The amount of lactic acid produced was nearly equal to the amount of glucose destroyed.

(4) The addition of either insulin or thyroxin produced no demonstrable effect upon the glucose metabolism.

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REFERENCES.

BLANCHETIÈRE, A.—(1927) *Bull. Ass. franç. Cancer*, **16**, 3.
CLAUSEN, S. W.—(1922) *J. Biol. Chem.*, **52**, 263.
CRAMER, W., DICKENS, F., AND DODDS, E. C.—(1926) *Brit. J. Exp. Path.*, **7**, 299.
FRIEDMANN, T. E., COTONIO, M., AND SHAFFER, P. A.—(1927) *J. Biol. Chem.*, **73**, 335.
MINAMI, S.—(1923) *Biochem. Z.*, **142**, 334.
STAHL, O., AND WARBURG, O.—(1926) *Klin. Wschr.*, **5**, 1218.
WARBURG, O.—(1925) *Biochem. Zeit.*, **160**, 307.—(1926) 'Über den Stoffwechsel der Tumoren,' Berlin (Julius Springer).—(1927[1]) *Biochem. Z.*, **184**, 484.—(1927[2]) *Naturwissenschaften*, **15**, 1.
WARBURG, O., POSENER, K., AND NEGELEIN, E.—(1924) *Biochem. Z.*, **152**, 309.
WIND, F.—(1926) *Biochem. Z.*, **179**, 384.

THE EFFECT OF HEAT ON O AND H IMMUNE BODIES.

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THE effect of heat on the immune bodies of the blood serum has been the subject of numerous investigations. Agglutinins especially have been often examined, with the view of testing the resistance to heat of agglutinins for various species of bacteria or of those for a particular micro-organism when examined in the blood serum of various species of animals. Normal agglutinins were also compared in this respect with immune agglutinins and these latter with the immune bodies concerned in other immunity reactions. The results obtained by various workers were often contradictory and on several of the points investigated opinions remained discrepant.

Streng (1909) studied very exhaustively the factors which determine the velocity of the inactivation by heat of agglutinins for *B. coli* and *B. typhosus*. The following were the most important conclusions reached by this worker: (1) That the inactivation of the agglutinating power does not occur at a precisely fixed temperature but occurs in a zone extending over many degrees centigrade (usually between the limits 60° and 80° C.). (2) That this zone does not vary with the serum of various species of animals but varies markedly

with the serum of individual animals of the same species. (3) That there are two types of this reaction which are independent of the micro-organism concerned. (4) That the type of reaction does not depend on the fluctuating amount of alkali, salt and protein, normally occurring in the blood serum. (5) That it appears necessary to assume the existence of a series of partial-agglutinins of different resistance to heat (analogous to the series of globulin fractions).

This thorough study by Streng as well as the older work by Pick (1902) and Eisenberg and Volk (1902)—who from the different heat-resistances of agglutinins for various bacterial species and in the serum of various species of animals concluded that agglutinins are associated with various fractions of the serum globulin—was made on bacterial species which are now known to possess two different kinds of agglutinogen with corresponding agglutinins. It is obvious that in the study of the heat resistance of agglutinins the qualitative differences obtaining in these substances must also be taken into consideration.

The earlier observations of Joos (1903), who, working with *B. typhosus* distinguished two kinds of antigen and antibody by their resistance to heat, did not withstand the test of criticism. Beyer and Reagh (1904) brought the observations of Joos into correlation with the important findings of Smith and Reagh (1903) on the non-identity of flagellar and somatic agglutinins for hog-cholera bacilli. They found that the flagellar and somatic agglutinins and agglutinable substances of the hog-cholera bacillus can be differentiated by heat. Buxton and Torrey (1906) working with *B. typhosus* found that the two agglutinins corresponding to their "stable" and "detachable" agglutinogens showed no perceptible difference in their resistance to heat. Smith and Ten Broeck (1914-1915) in their comparative study of fowl typhoid and *B. typhosus* were not able "to distinguish between somatic and flagellar agglutinins by the use of heat as was possible in the experiments of Beyer and Reagh made with two strains of hog-cholera bacilli." It is now obvious that these divergent results were due to the low temperature used by all these workers, a temperature which did not allow of a differentiation of the two antigens.

B. proteus X_{19} agglutinins in the serum of typhus patients were shown by Hamburger and Bauch (1917) and Csepai (1917) to be much less resistant to heat than those in the serum of rabbits inoculated with that organism, the reason for this, as demonstrated by Weil and Felix (1917) by means of their O and H cultures, being that O and H agglutinins possess different heat-resistances. Prausnitz (1920) and other workers suggested the use of this distinct difference as an auxiliary means for differentiation between the heat-labile O agglutinin in the serum of typhus patients and the heat-resistant H agglutinin in the serum of patients suffering from various proteus infections.

In *B. typhosus* and the Salmonella group of organisms the behaviour of O and H agglutinins has not yet been adequately investigated. In Balteanu's (1926) careful work on *Vibrio choleræ* this method of differentiation has already been usefully applied.

The following experiments were undertaken with the view of investigating in more detail the effect of heat on O and H agglutinins and its bearing on certain controversial problems.

Method of Manipulation of Serum Samples Used in These Experiments.

Since the work of Pick (1902) and of Eisenberg and Volk (1902) it is known that the coagulation of serum globulins very markedly interferes with the direct inactivating effect of heat on the agglutinins. Pick (1902) showed that agglutinins for *B. typhosus* in horse serum withstood even boiling for a short time, if by addition of urea or formalin the globulins had been prevented from coagulating. Eisenberg and Volk (1902) found that both these substances themselves impaired the agglutininophore group of the agglutinin. Eisenberg (1906) established in addition that diluting the serum increases the heat resistance of the agglutinin. Streng (1909), while disagreeing on some points, confirmed this statement of Eisenberg.

In Table I the heat resistance of agglutinins as affected by serum dilution is shown in the case of one H and of one O serum.

TABLE I.—*The Heat-Resistance of Agglutinins as Affected by Serum Dilution.*

Serum.	Serum dilution heated.	Type of agglutination.	Agglutination with the homologous strain. Titre of agglutination and the percentage that remains after heating the serum for 45 minutes at—						
			50°.	55°.	60°.	65°.	70°.	75°.	80°.
Rabbit serum, *B. enteritidis* Gaertner 60° C. (H serum)	1:10	H	1:30,000	1:30,000	1:20,000	1:10,000	1:5000	1:1000	<1:50
			100	100	66	33	16·6	3·3	0
	1:1000	H	1:30,000	1:30,000	1:30,000	1:20,000	1:15,000	1:10,000	1:5000
			100	100	100	66	50	33	16·6
Rabbit serum, *B. paratyphosus* A 100° C. (pure O serum)	1:10	O	1:5000	1:5000	1:3000	1:200	<1:50	<1:50	<1:50
			100	100	66	4	0	0	0
	1:500	O	1:5000	1:5000	1:5000	1.2000	<1:500	<1:500	<1:500
			100	100	100	40	0	0	0

Serum dilutions 1 : 10 after heating at 50°–65° C., clear; after heating at 70°, 75° and 80° C., turbid (from opalescence to intense turbidity).
Serum dilutions 1 : 1000 and 1 : 500 after heating up to 80° C., clear.
Agglutination with fresh saline suspensions of living bacteria. Reading after 24 hours (2 hours' incubation at 37° C., then at room temperature). Titre = highest dilution showing traces of agglutination (estimated by means of a magnifying lens).

Apart from the striking difference between the low resistance of the O agglutinin (for *B. paratyphosus A*) and the high resistance of the H agglutinin (for *B. enteritidis* Gaertner) the effect of diluting the serum is clearly brought out in Table I. This effect can be better demonstrated with the H agglutinin, whose higher resistance permits the observation of the phenomenon up to the temperature of 80° C. But in O agglutination too the comparison of the titre and the percentage of the agglutinin that remains intact after heating at 60° and 65° C. in the dilutions 1 : 10 and 1 : 500 respectively clearly indicates the effectiveness of this procedure.

It is not proposed to discuss here the mechanism of the phenomenon and its bearing on the complicated problem of the assumed association of immune body and serum globulins. It may, however, be mentioned in passing that as regards inactivation of agglutinins by exposure to ultra-violet rays diluting the serum has exactly the opposite effect (Baroni and Jonesco-Mihalesti, 1910; Heuer, 1922).

In the following experiments the sera were always exposed to the action of

heat in the highest possible dilution in order to reduce as far as possible any interference by the altered globulins. With the exception of the experiment reproduced in Table I the dilutions used were high enough to prevent the occurrence of any visible change due to coagulation. As heating to 50° C. has in no instance shown any effect on the titre, an unheated control, although carried out in each experiment, has not been included in the tables.

HEAT RESISTANCE OF IMMUNE O AND H AGGLUTININS FOR VARIOUS BACTERIAL SPECIES.

In Table I an illustration has already been given of the low resistance of O agglutinins and the high resistance of H agglutinins in *B. paratyphosus A* and *B. enteritidis* Gaertner respectively. It was proved in numerous experiments with rabbit immune sera containing O and H agglutinins for the homologous strain in approximately equal concentrations that this qualitative difference is met with in all members of the typhoid-paratyphoid group of organisms. In order to compare this group with *B. proteus X* strains, previously investigated by Weil and Felix (1917), rabbits were immunized by simultaneous or consecutive inoculation with two or more organisms. If this procedure did not yield a serum which reacted with each of the strains concerned to a titre suitable for comparison, resource was had to mixing monovalent rabbit sera in appropriate proportions. Table II shows an experiment with a mixed serum.

TABLE II.—*Heat Resistance of Immune O and H Agglutinins for* B. typhosus, B. enteritidis Gaertner *and* B. proteus X.

Agglutination with strain.	Type of agglutination.	Rabbit serum No. 140 (*B. enteritidis* Gaertner 60°) and rabbit serum No. 163 (*B. proteus* X_{19} 60°) mixed in the proportion 1 : 3. Titre of agglutination and the percentage that remains after heating the mixture in a dilution 1 : 100 for 45 minutes at—					
		50°.	55°.	60°.	65°.	70°.	75°.
B. typhosus 901	O	1 : 10,000	1 : 10,000	1 : 5000	1 : 3000	<1 : 100	<1 : 100
		100	100	50	30	0	0
B. proteus O X_{19}	O	1 : 5000	1 : 5000	1 : 3000	1 : 1500	<1 : 100	<1 : 100
		100	100	60	30	0	0
B. proteus H X_2	H	1 : 2000	1 : 2000	1 : 2000	1 : 1500	1 : 200	1 : 100
		100	100	100	75	10	5
B. enteritidis Gaertner G_3	H	1 . 5000	1 : 5000	1 : 5000	1 : 3000	1 : 500	1 : 200
		100	100	100	60	10	4

It is seen from Table II that the conformity of results is perfect. Each kind of agglutinin has its own degree of heat-resistance irrespective of the organism to which it corresponds. Attention is drawn to the fact that sera have purposely been chosen with H titres lower than their corresponding O titres, in order to emphasize the qualitative character of the phenomen.

In similar experiments the purely stabilotropic O agglutinins for some species which do not possess the double type of antigen and antibody were compared with those for the typhoid-paratyphoid group of organisms. The agglutinin for *B. melitensis* was found to possess a resistance equal to that of

the O agglutinins for *B. typhosus*, whereas those for *B. dysenteriæ* Flexner and Y appeared to be somewhat more resistant but still far less so than the H agglutinins.

The variation in resistance to heat of agglutinins in the serum of individual animals of the same species, to which Streng (1909) and Madsen and Streng (1910) attached such great importance, was not met with in our experiments. It seems very likely that the individual variations so carefully observed by Streng can be satisfactorily explained by the varying amount of H and O agglutinins contained in his *B. typhosus* and *B. coli* immune sera.

HEAT-RESISTANCE OF NORMAL AND IMMUNE AGGLUTININS.

The different resistance to the effect of heat observed in normal and immune agglutinins has led to considerable discrepancies in the opinions held by various authors concerning the relationship between normal immune bodies and those due to immunization. The observations of Rodet (1903), Eisenberg (1906) and Lüdke (1906) on bacterial agglutinins and especially those of Landsteiner and Reich (1905) and Prašek (1913) on hæmagglutinins are mostly quoted in support of Landsteiner's theory, that the immune bodies of the normal serum differ qualitatively from those produced in the course of immunization. Streng (1909) working with *B. coli* and Petrotta (1921) working with *B. typhosus* found no difference in heat resistance between normal and immune agglutinins.

Schiff (1924) rightly emphasized that, as far as bacterial agglutinins are concerned, these observations need re-investigation in which attention should be paid to the two types of agglutinins, for which he (1922) had suggested the nomenclature stabilotropic (O) and labilotropic (H). In human beings as well as in some species of animals Schiff (1922) had found only normal agglutinins of the stabilotropic O type. In normal cow serum, however, which is known to be very rich in various normal serum substances, the presence of both O and H agglutinins has been demonstrated by Breinl (1920). From our experience we may state that occasionally the same is found to be the case with normal horse serum. Schiff's (1924) demand that as regards heat-resistance the pure O agglutinin of a normal serum should be compared only with the corresponding O component of an immune serum, is therefore fully justified.

TABLE III.—*Normal and Immune O Agglutinins for* B. typhosus *(in Rabbit Serum).*

	Type of agglutination.	Agglutination with strain *B. typhosus* 901. Titre of agglutination and the percentage that remains after heating the serum in a dilution 1 : 25 for 45 minutes at—					
		50°.	55°.	60°.	65°.	70°.	75°.
Serum 1	O	1 : 100	1 : 100	1 : 75	1 : 50	<1 : 25	<1 : 25
		100	100	75	50	0	0
Serum 2	O	1 : 200	1 : 200	1 : 150	1 : 100	<1 : 25	<1 : 25
		100	100	75	50	0	0

Serum 1 = normal serum, rabbit No. 159.
Serum 2 = immune serum, rabbit No. 139 (*B. typhosus* 100° C.) diluted in the proportion 1 : 25 with the normal serum of rabbit No. 161, which did not contain agglutinins for *B. typhosus* 901 even in a dilution 1 : 20.

In Table III the O agglutinin for *B. typhosus* of a normal rabbit serum is compared with the O agglutinin of a pure O immune serum. A necessary condition for an experiment of this kind is as follows: the serum dilutions to be exposed to heat must contain not only equal amounts of agglutinin, but also equal quantities of serum globulin. Realizing this, Eisenberg (1906) has already suggested the dilution of immune serum with a normal serum of the same species, but as far as we are aware neither he nor any other worker has applied this procedure. We have used it in our experiments and did not encounter much difficulty in selecting normal rabbits whose serum was sufficiently poor in agglutinins to serve as the medium for diluting the high titre immune serum.

It is seen from Table III that no difference whatever could be established between the resistance of the normal and immune O agglutinin. The titre of the immune agglutinin was purposely left slightly the higher, in order to give the experiment still more weight.

Immune and normal agglutinins, present in rabbit serum, for *B. dysenteriæ* Flexner, and *Y* were tested in exactly the same way. The result was uniformly the same as that shown in Table III.

On the other hand the normal O and H agglutinins present in cow serum were found to behave in exactly the same way as the corresponding immune agglutinins. Such an experiment is illustrated by Table IV.

TABLE IV.—*Normal O and H Agglutinins for* B. proteus X *in Cow Serum.*

Agglutination with strain.	Type of agglutination.	Titre of agglutination and the percentage that remains after heating normal cow serum in a dilution 1:20 for 45 minutes at—					
		50°.	55°.	60°.	65°.	70°.	75°.
B. proteus H X_{19}	H	1:80	1:80	1:80	1:60	1:20	<1:20
		100	100	100	75	25	0
B. proteus O X_2	O	1:150	1:150	1:60	1:20	<1:20	<1:20
		100	100	40	13·3	0	0

The conclusion therefore appears justified that, as far as bacterial agglutinins are concerned, the assumed qualitative difference in heat-resistance of normal and immune agglutinins does not exist but has hitherto been mistakenly suggested by the qualitative difference between the O and H immune bodies. Considerable evidence has already been accumulated to indicate the decisive role which is played by the O immune body in complement fixation (Weil and Felix, 1920; Weil, 1921; Felix and Robertson, 1928) and in bactericidal and opsonic serum actions (Felix, 1924; Braun and Nodake, 1924; Felix and Olitzki, 1926; Lubinski, 1928). It may therefore be assumed that the conclusion reached as regards normal and immune agglutinins is valid for normal and immune antibodies generally.

THE PECULIAR HEAT-LABILITY OF *B. PROTEUS* X_{19} AGGLUTININS IN THE SERUM OF TYPHUS PATIENTS.

The striking heat-lability of the X_{19} agglutinin in the serum of typhus patients attracted—as already mentioned—the attention of numerous workers. The principle involved, *i.e.* the qualitative difference between O and H

agglutinins had been established by Weil and Felix (1917) but there remained still a peculiarity which needed further investigation.

When the heat-resistance of the O agglutinin in typhus patients' serum is compared with that in the serum of rabbits inoculated with X_{19} it is uniformly found that the former is less resistant than the latter. The difference is clearly illustrated in Table V.

TABLE V.—*Immune O Agglutinins for* B. proteus X_{19} *in the Serum of Typhus Patients and in Rabbit Immune Serum.*

Serum.	Agglutination with strain.	Type of agglutination.	Titre of agglutination and the percentage that remains after heating the sera in a dilution 1 : 50 for 40 minutes at—					
			50°.	55°.	60°.	65°.	70°.	75°.
Rabbit serum *B. proteus* H X_{19} 60°	H X_2	H	1:2000	1:2000	1:2000	1:1000	1:500	1:200
			100	100	100	50	25	10
	O X_{19}	O	1:5000	1:5000	1:4000	1:1000	<1:50	<1:50
			100	100	80	20	0	0
Typhus patient No. 1	O X_{19}	O	1:5000	1:2000	1:1000	<1:50	<1:50	<1:50
			100	40	20	0	0	0
Typhus patient No. 2	O X_{19}	O	1:10,000	1:5000	1:2000	<1:50	<1:50	<1:50
			100	50	20	0	0	0

In table V. the relationship between the heat-resistances of the O and H agglutinin in the rabbit serum is the same as has been demonstrated in previous experiments. But in the two sera from typhus patients the O agglutinin is markedly less resistant than it is in the rabbit serum.

A difference in the coagulation temperatures of human and rabbit serum proteins was at first considered as one of the possible explanations. But in experiments comparing human typhoid sera with *B. typhosus* immune sera from rabbits no difference could be demonstrated in the heat-resistances of their respective H and O agglutinins. On the other hand the H agglutinins for *B. proteus* X_{19} in human serum (from a patient suffering from pyelonephritis due to infection with *B. proteus* group III; Weil and Felix, 1918) were found to equal in heat-resistance those in rabbit serum.

TABLE VI.—*H and O Immune Agglutinins for* B. proteus *and* B. typhosus *in Human Serum.*

Serum from patients suffering from—	Agglutination with strain.	Type of agglutination.	Titre of agglutination and the percentage that remains after heating the sera in a dilution 1 : 50 for 40 minutes at—					
			50°.	55°.	60°.	65°.	70°.	75°.
Typhoid fever (O serum)	*B. typhosus* strain 901	O	1:500	1:500	1:400	1:100	<1:50	<1:50
			100	100	80	20	0	0
Typhus fever (O serum)	*B. proteus* H X_{19}	O	1:5000	1:3000	1:2000	<1.50	<1:50	<1:50
			100	60	40	0	0	0
Typhoid fever (H serum)	*B. typhosus* strain 901	H	1:2000	1:2000	1:2000	1:1000	1:200	1.50
			100	100	100	50	10	2·5
Proteus pyelonephritis (H serum)	*B. proteus* H X_{19}	H	1:5000	1:5000	1:5000	1:2000	1:500	1:100
			100	100	100	40	10	2

In Table VI sera from patients suffering from various diseases are compared in this respect. It is only the X_{19} agglutinin in the typhus patient's serum which shows a heat-resistance markedly lower than that described for O agglutinins in previous experiments with rabbit sera.

It was further found that in several sera from rabbits infected with typhus virus (according to Weil and Felix, 1921), the O agglutinin for X_{19} did not differ in heat-resistance from the O agglutinin in rabbits inoculated with that organism. Therefore an agglutinogenic difference between the OX_{19} antigen in typhus virus and that present in *B. proteus* X_{19} does not seem to be responsible for the remarkable heat-lability of the O agglutinin in typhus patients' serum.

The most likely reason for the phenomenon under discussion appears to be the marked alteration of globulins which is often met with in the patients' serum at the height of the typhus infection and during convalescence. For instance alteration of globulins can be easily demonstrated by the so-called Weltmann reaction (1916) (*i.e.* the dilution of serum with distilled water 1 : 10 produces turbidity), and is further probably associated with the positive Wassermann reaction described in typhus fever by various workers. As has been pointed out in a previous section of this paper, the globulin content of the serum markedly influences the heat destruction rate of agglutinins.

H AND O AGGLUTININS IN RELATION TO COMPLEMENT-FIXATION AND BACTERICIDAL SERUM ACTION.

An attempt was made to use the different heat-resistances of the H and O agglutinins in order to demonstrate their relationship to the immune bodies concerned in complement-fixation and in bactericidal serum action. Such experiments were carried out with *B. typhosus* and *B. paratyphosus A*. An example is given in Table VII.

TABLE VII.—*H and O Agglutinins in Relation to Complement Fixation and Bactericidal Serum Action.*

Immune serum: rabbit serum *B. paratyphosus* A 60°.
Titre and the percentage that remains after heating the serum.

Serum heated in a dilution 1 : 50 for 45 minutes at—	H. agglutination.		O. agglutination.		Complement fixation.		Bactericidal action *in vitro*.	
	Titre.	%.	Titre.	%.	Titre.	%.	Titre.	%.
50°	1 : 5000	100	1 : 500	100	1 : 400	100	1 : 100,000	100
55°	1 : 5000	100	1 : 500	100	1 : 400	100	1 : 100,000	100
60°	1 : 5000	100	1 : 400	80	1 : 300	75	1 : 75,000	75
65°	1 : 3000	60	1 : 100	20	1 : 50	12·5	1 : 20,000	20
70°	1 : 500	10	< 1 : 50	0	< 1 : 50	0	< 1 : 100	0
75°	1 : 200	4	< 1 : 50	0	< 1 : 50	0	< 1 : 100	0
80°	1 : 50	1	< 1 : 50	0	< 1 : 50	0	< 1 : 100	0

Technique of complement fixation, see Felix and Robertson (1928).
Technique of bactericidal action *in vitro*, see Felix and Olitzki (1926).

It is seen from Table VII that, within the limits of experimental error, the titres of the heated serum in O agglutination, complement-fixation and bactericidal serum action *in vitro* fairly satisfactorily correspond with each other, while those in H agglutination run an independent course. This

finding can serve as additional evidence in support of the view that the O immune body only is concerned in complement-fixation and in bactericidal serum action.

SUMMARY.

(1) O and H agglutinins for *B. typhosus* and the Salmonella group of organisms possess heat-resistances as markedly different from each other as those already described in the case of the *B. proteus X* strains.

(2) This constant property of the two kinds of agglutinins is additional evidence of the qualitative difference between the two types of bacterial antigens and immune bodies.

(3) Its application as a diagnostic measure, while useful in the special case of typhus patients' serum, does not appear to be of importance in other infections.

(4) Normal and immune agglutinins possess the same resistance to heat.

(5) The close relationship between complement binding and bactericidal immune bodies and O agglutinins, already established by other means, is also demonstrated by their similar heat resistances.

REFERENCES.

Balteanu, J.—(1926) *J. Path. Bact.*, **29**, 251.
Baroni, V. and Jonesco-Mihalesti, C.—(1910) *C.R. Soc. Biol.*, Paris, **68**, 393.
Beyer, H. G., and Reagh, A. L.—(1904) *J. Med. Res.*, **12**, 313.
Braun, H., and Nodake, R.—(1924) *Zbl. Bakt.*, **92**, 429.
Breinl, F.—(1920) *Z. ImmunForsch.*, **29**, 491.
Buxton, B. H., and Torrey, J. C.—(1906) *J. Med. Res.*, **14**, 527.
Csepai, K.—(1917) *Wien. klin. Wschr.*, p. 1264.
Eisenberg, Ph.—(1906) *Zbl. Bakt.*, **41**, 96.
Eisenberg, Ph., and Volk, R.—(1902) *Z. Hyg.*, **40**, 155.
Felix, A.—(1924) *J. Immunol.*, **9**, 115.
Felix, A., and Olitzki, L.—(1926) *Ibid.*, **11**, 31.
Felix, A., and Robertson, M.—(1928) *Brit. J. Exp. Path.*, **30**, 345.
Hamburger, R., and Bauch, R.—(1917) *Deut. med. Wschr.*, p. 1130.
Heuer, G.—(1922) *Zbl. Bakt.*, **88**, 380.
Joos, A.—(1903) *Ibid.*, **33**, 762.
Landsteiner, K., and Reich, M.—(1905) *Ibid.*, **39**, 712.
Lubinski, H.—(1928) *Ibid.*, **106**, 200.
Lüdke, H., (1906) *Ibid.*, **42**, 260.
Madsen, Th., and Streng, O.—(1910) *Z. phys. Chem.*, **70**, 263.
Petrotta.—(1921) *Ann. Clin. med.*, **10**, 425.
Pick, E. P.—(1902) *Beitr. chem. Physiol. Path.*, **1**, 350.
Prašek, E.—(1913) *Z. ImmunForsch.*, **20**, 146.
Prausnitz, C.—(1920) *Zbl. Bakt.*, **84**, 103.
Rodet, M. A —(1903) *C.R. Soc. Biol.*, Paris, **55**, 1628.
Schiff, F.—(1922) *Z. ImmunForsch.*, **33**, 511.—(1924) Oppenheimer's 'Handbuch der Biochemie,' Ed. II, **3**, 262, Jena.
Smith, Th., and Reagh, A. L.—(1903) *J. Med. Res.*, **10**, 89.
Smith, Th., and Ten Broeck, C.—(1914-1915) *Ibid.*, **31**, 503.
Streng, O.—(1909) *Z. Hyg.*, **62**, 281.
Weil, E.—(1921) *Z. ImmunForsch.*, **31**, 50.
Weil, E., and Felix, A.—(1917) *Wien. klin. Wschr.*, p. 1509.—(1918) *Ibid.*, p. 637.—(1920) *Z. ImmunForsch.*, **29**, 24.—(1921) *Ibid.*, **31**, 457.
Weltmann, O.—(1916) *Wien. klin. Wschr.*, p. 573.

CULTIVATION OF VACCINIA VIRUS.

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THE cultivation of vaccinia virus in conjunction with growing tissue has been studied to a considerable extent. Parker (1923–24) and Parker and Nye (1925) were successful in propagating the virus through eleven generations and obtained an increase of 51,000 times. Small pieces of vaccine—infected rabbit's testis with rabbit's plasma were used and active proliferation of tissue took place regularly.

Craciun and Oppenheimer (1926) separated the granular bodies described by Paschen (1906) from glycerinated calf-lymph. They claim to have cultivated these in embryonic guinea-pig cornea with guinea-pig plasma and Lock's solution on inverted coverslips in hollow-ground glass slides. These bodies survived for seventy-one days providing the cultures showed actively growing cells, but failed to survive if cultivated with dying or dead cells. This work has not yet been confirmed.

Carrel and Rivers (1927) described experiments in which they obtained increase of the virus using finely minced chick embryo tissue. A strain of testicular virus was fixed to the tissue by standing for a period at cold room temperature. This virus-pulp emulsion was then mixed with Tyrode's solution and hen-plasma in suitable proportions and poured into Carrel "D" type culture flasks where clotting took place. After periods of incubation, varying from eight days to one month, the contents of these flasks were ground in a mortar and the supernatant fluid, suitably diluted, injected intradermally in rabbits. These tests showed an increase of 400 times after eight days' incubation. The technique was simple, and, in the opinion of the authors, could be adapted readily to the industrial production of vaccinia virus.

Haagen (1928) cultivated the virus, using rabbit spleen and plasma in which was incorporated a very small piece of rabbit testicle. These were placed on mica coverslips over hollow-ground slides and incubated for three days, after which they were immersed for two or three minutes in a virus-containing fluid. The testicular implants were then placed in fresh plasma-spleen mixture and re-incubated. Thirty-seven subcultures were made and a thousandfold increase in virus obtained.

In view of these investigations it was decided to study the problem more fully and to learn, if possible, something of the factors involved in the cultivation of viruses generally. The advantages of an apparently simple laboratory method of obtaining large quantities of vaccinia virus free from bacteria and tissue *débris* are obvious.

METHODS.

Our methods were based upon those employed by Carrel and Rivers. The materials used are described first, then the technique of their employment.

Virus.—The vaccinia virus used as "seed" in these experiments was obtained from the excised dermal passage lesion of a testicular virus supplied by Dr. Rivers. The dermal reactions were excised on the fourth or fifth day after injection of the virus, ground with sand, and 2 to 3 c.c. of M/50 sodium phosphate (pH 7·4) added. The supernatant obtained after centrifuging gave an abundant yield of sterile virus. This method of propagating the virus has been described in detail (Ledingham and McClean, 1928). It was found that the pH of phosphate solution made up in the following proportions remained steady:

Sodium dihydrogen phosphate (NaH_2PO_4) M/50.
Disodium hydrogen phosphate (Na_2HPO_4) M/50.

When 150 c.c. of this solution of (NaH_2PO_4) are added to 1 litre of the solution of (Na_2HPO_4) the required pH is obtained.

Tissue.—Hatching eggs were incubated at 39°–40°C. for seven to ten days. After the first 48 hours the eggs are turned once a day. Since these eggs are very sensitive to small variations of temperature and to vibration it is necessary to guard against these factors. The embryos were the source of tissue for culture and of the embryo extract with which these cultures were fed periodically.

The eggs are opened with aseptic precautions in a tissue culture box. The round end of the egg is swabbed with alcohol and the shell pierced with forceps. The air-space is thus exposed; the shell covering this is removed and, using fresh forceps, the thick white membrane is detached. This exposes the amniotic sac through which the embryo can be seen. This sac is snipped with scissors and the whole contents of the egg turned out into a Petri dish. The embryo, freed from the membranes, is then lifted clear with a teaspoon and placed in another Petri dish. In this way as many embryos as required may be collected in one dish. This method of obtaining the embryos is more convenient than the technique described by Carrol and Rivers and does not increase the risk of contamination.

The embryos are washed with Tyrode's solution, to remove traces of yolk and albumen, and minced up as finely as possible with curved, pointed scissors. The further treatment of this embryo pulp will be described under the appropriate headings.

In some of our experiments the brain-tissue of fourteen-day embryos was cultured. The embryos are decapitated and the heads placed in a dish. After washing with Tyrode's solution the skulls are opened along the sagittal suture with scissors and the skull-cap turned back as two flaps. The brains can then be removed with curved forceps and minced up with scissors.

Embryo extract.—The embryo pulp of 7–10-day chicks is centrifuged, the supernatant fluid removed aseptically and diluted 1 : 20 in Tyrode's solution. This extract is tested for sterility and stored in the cold room where it will keep satisfactorily for a week. At least 2 c.c. of each batch of extract should be tested for sterility in ordinary nutrient broth and incubated for at least 48 hours.

Tyrode's solution.—The solution used for these experiments was made up according to the following formula as modified by Robison for his work on calcification *in vitro*:

NaCl	6	gm.
KCl	0·4	,,
$MgSO_4$	0·25	,,
KH_2PO_4	0·18	,,
$CaCl_2$	0·275	,,
Glucose	1·0	,,
Distilled water to 1 litre		

The above solution was adjusted to pH 7·3 with standard NaOH solution.

This solution is sterilized by filtration through a Seitz filter under slight positive pressure into a "Pyrex" filter-flask fitted with resistance glass tubing and a hooded pipette. After filtration, sterility and pH tests are made, and the solution stored in the cold room.

Plasma.—In the earlier experiments plasma was obtained by bleeding a hen from the wing-vein into a sterile syringe containing 0·4 mgm. of heparin in 1 c.c. of sterile saline. This was done immediately before each experiment. Later, it was found to be more satisfactory to bleed out a cock under anæsthesia from the sciatic artery and store the plasma in the cold room until required. The artery is exposed from the external aspect; the vessel is cleaned and any branches tied. The distal end of the exposed position is ligatured and the proximal end seized with a small "bulldog" clip. The artery is then opened with fine pointed scissors and the isolated portion washed out with sterile saline through a capillary pipette. A glass cannula is inserted, tied in position and the "bulldog" clip released. The first few c.c. of blood are discarded. The tubes in which the blood is collected contain heparin dissolved in saline which has been sterilized in the autoclave for 15 minutes at 15 lb. pressure. In our experience 1·2 mgm. of heparin in 3 c.c. of saline has been sufficient to prevent clotting in all the blood that can be obtained from a cock. This gives a final concentration of heparin of about 1 : 8000 to 1 : 10,000 according to the yield of the blood. The tubes of blood are placed in ice immediately. When cool, the blood is transferred to sterile centrifuge tubes, centrifuged, and the plasma pipetted off. This plasma should be tested for sterility before use. It may be stored in the cold room for several weeks, and after this period will clot when brought into contact with fresh tissue and will allow tissue growth to take place in the resulting coagulum.

Method of setting up cultures.—Embryo pulp is mixed with equal amounts of Tyrode's solution and "seed" virus, diluted so as to be potent at about 1 : 1000. This virus-pulp emulsion is placed in the cold room for 1¼ hours to fix the virus to the tissue. After this interval the emulsion is further diluted with Tyrode, plasma added and the mixture distributed rapidly before clotting has taken place in 2 c.c. amounts into Carrel's "D" type culture flasks.

The actual amounts used were: 1 c.c. embryo pulp; 1 c.c. Tyrode's solution; 1 c.c. virus diluted in Tyrode.

After fixation in the cold room the following mixture is made: 1 c.c. virus-pulp emulsion; 6 c.c. Tyrode's solution; 3 c.c. plasma.

This gives 10 c.c. of inoculum, which is sufficient for five flasks and is a final dilution of the virus of 1 : 30. Clotting should take place within two to three minutes of the addition of the plasma. The mouths of the flasks are then flamed and capped and the flasks incubated at 37°C. Sterility tests are made on the virus-pulp emulsion, plasma and Tyrode at the time of setting up the experiments in order that any contamination which may appear can be traced to its source.

One culture flask is set aside as a control and the inoculum titrated intradermally on a rabbit for activity of virus. This control may be titrated immediately, after eighteen hours at 37° C. or after a similar period in the cold room; we were unable to detect any material difference in the titre whichever of the above methods was adopted. The distribution of tissue in the culture flasks is not even throughout any one batch, but no appreciable difference of titre in those controls containing maximum or minimum amounts of tissue could be detected. The method of titrating the yield of virus in any culture flask will be described later in detail.

The cultures are fed every two or three days. After removing any free supernatant fluid with a capillary pipette 2 c.c. of embryo extract are added to each flask. This extract is warmed in the incubator at 37°C. before use. It was found more satisfactory to replace the old cotton-wool plugs with fresh ones taken from clean sterilized culture flasks at each feeding; this prevents gradual disintegration of the plugs as the cultures proceed. Sterilized rubber corks, as a substitute for wool plugs and rubber caps, are not satisfactory; moisture condenses between the neck of the flask and the cork and invites contamination.

In some of our experiments bulk cultures were set up in triangular Roux flasks which held 40–50 c.c. of inoculum. The various ingredients were used in the same proportions as those described above. The tissue grows satisfactorily in these flasks which give a large yield of inoculum, the titre of which can be estimated by one titration. On the other hand, there is increased risk of contamination, which is the more disastrous in that the whole bulk of the inoculum may be destroyed instead of the small amount in any one culture flask.

Titration of virus in culture flasks.—The escape of virus into the free fluid being negligible the latter is removed and discarded. The coagulum is then detached with a stout platinum loop and transferred to a mortar in a tissue-culture box. A small amount of sand is added and the mixture ground up. The resulting fluid is centrifuged, and the supernatant, after removal, made up to the same volume as that obtained from the control flask. This volume is usually about 1·4 c.c.; the yield of fluid diminishes with the age of the cultures, and, if this is not corrected by dilution with phosphate, will produce a relative concentration of the virus.

Suitable dilutions are made in saline and these titrated in 0·2 c.c. amounts, intradermally on the back of a rabbit which has been shaved, or depilated the previous day. Results may be read from the fourth to the sixth day; later readings are untrustworthy owing to the occasional appearance of a scattered generalized eruption. Several inocula may be titrated on the same rabbit.

RESULTS.

Table I shows the results of three consecutive culture experiments, all made directly from the seed dermal virus. Owing to technical difficulties which were only eliminated by experience, the nine preceding experiments failed, or were only partially successful. In each of these experiments two control flasks were titrated, one containing a maximum and the other a minimum amount of tissue. No appreciable difference in titre could be detected. The cultures, potent at 1 : 20,000 were possibly active at a higher dilution, but titrations were not carried further. As will be seen in Tables II and III, considerably higher titrations were made in later experiments. The free supernatant fluid, due to feeding with embryo extract, in flasks potent at 1 : 20,000 was titrated and found to be potent at 1 : 10 or 1 : 100. Escape of virus into this fluid is, therefore, negligible.

In Experiments 10 and 11, the best flasks showed a two-thousandfold multiplication of virus. In both experiments, flasks showing coagulum in good condition with active tissue-growth were incubated until the twelfth and thirteenth days, but on titration showed no increase of virus. That this was not due to prolonged incubation is shown by the high yield of virus obtained from one flask at a later date. On the contrary some flasks, in which the coagulum had disintegrated and tissue-growth ceased owing to late bacterial contamination, gave a maximum yield of virus.

In Experiment 12 the tissue was cultured in a Roux flask. There was a thousandfold increase of virus in a yield of 25 c.c. of material.

Table II summarizes the results of subculture experiments. The date of each experiment is given. The titre of control and culture flasks is expressed in terms of the highest dilution at which they produced a dermal reaction in the rabbit. The figure under "Increase" represents the titre of those flasks showing the maximum increase in any one set of cultures.

In whole embryo cultures the virus was successfully carried through two generations, but died out in the third subculture on three occasions. In brain-tissue this virus, which has died out in the whole embryo, gave good multiplication and was carried on successfully through two more generations. These subcultures were not carried further than the fourth generation owing to unexpected deterioration of the culture virus on storage during an interval between experiments. Subcultures in brain-tissue direct from the first whole embryo culture gave good multiplication through two subcultures, but died out in the third generation, probably owing to early gross bacterial contamination which liquefied the coagulum and destroyed the tissue.

Cultures in embryonic eye-tissue gave sporadic increase of virus in one or two flasks of each set of cultures. There was quite satisfactory tissue-growth in these cultures.

Table III shows whole embryo and brain-cultures compared in parallel vertical columns. Those experiments showing control flasks of approximately equal titres are placed opposite each other for comparison.

"At least" after the titration figure signifies that the titration was not carried to a higher dilution than the one shown, in which the virus was still active.

TABLE I.

	Experiment No. 10.	Experiment No. 11.	Experiment No. 12. Bulk culture in Roux flask and four culture flasks.
Control culture flasks	Potent neat Not potent at 1 : 10	Potent at 1 : 10 Not potent at 1 : 100	Potent at 1 : 10 Not potent at 1 : 100
Culture flasks after 5 days' incubation	...	...	(1) Potent at 1 : 10,000 (2) ,, 1 : 1000
Culture flasks after 6 days' incubation	(1) Potent at 1 : 10,000 (2) ,, 1 : 100	(1) Potent at 1 : 5000 (2) ,, 1 : 20,000 at least	...
Culture flasks after 7 days' incubation	(3) ,, 1 : 20,000 at least	...	(3) Potent at 1 : 1000 (4) ,, 1 : 5000
Culture flask after 8 days' incubation	(4) ,, 1 : 20,000 at least (5) ,, 1 : 20,000 (6) ,, 1 : 10,000 (7) ,, 1 : 5000	(3) Potent at 1 : 20,000 at least (4) ,, 1 : 20,000 at least	Roux flask— Potent at 1 : 10,000
Culture flasks after 9 days' incubation	(8) ,, 1 : 20,000 at least (9) ,, 1 : 20,000	(5) ,, 1 : 20,000 (6) ,, 1 : 5000	...
Culture flasks after 11 days' incubation	...	(7) ,, 1 : 10,000 ** (8) ,, 1 : 100 **	...
Culture flasks after 12 days' incubation	(10) Potent at 1 : 20,000 at least	(9) Not potent neat *	...
Culture flasks after 13 days' incubation	(11) Not potent neat *	(10) Potent at 1 : 20,000 ** at least	...
Culture flasks after 16 days' incubation	(12) ,, ,, ,, (13) ,, ,, ,,	...	...

Notes.—* Flasks showing coagulum in good condition with active tissue-growth.

** Flasks contaminated and the coagulum liquefied. Free supernatant, therefore, could not be removed and the titration represents a double dilution.

"At least" after titration figure signifies that the titration was not carried to a higher dilution than the one shown, in which the virus was still potent.

a double dilution.

"At least" after titration figure signifies that the titration was not carried to a higher dilution than the one shown, in which the virus was still potent.

TABLE II.—*Subculture Experiments.*

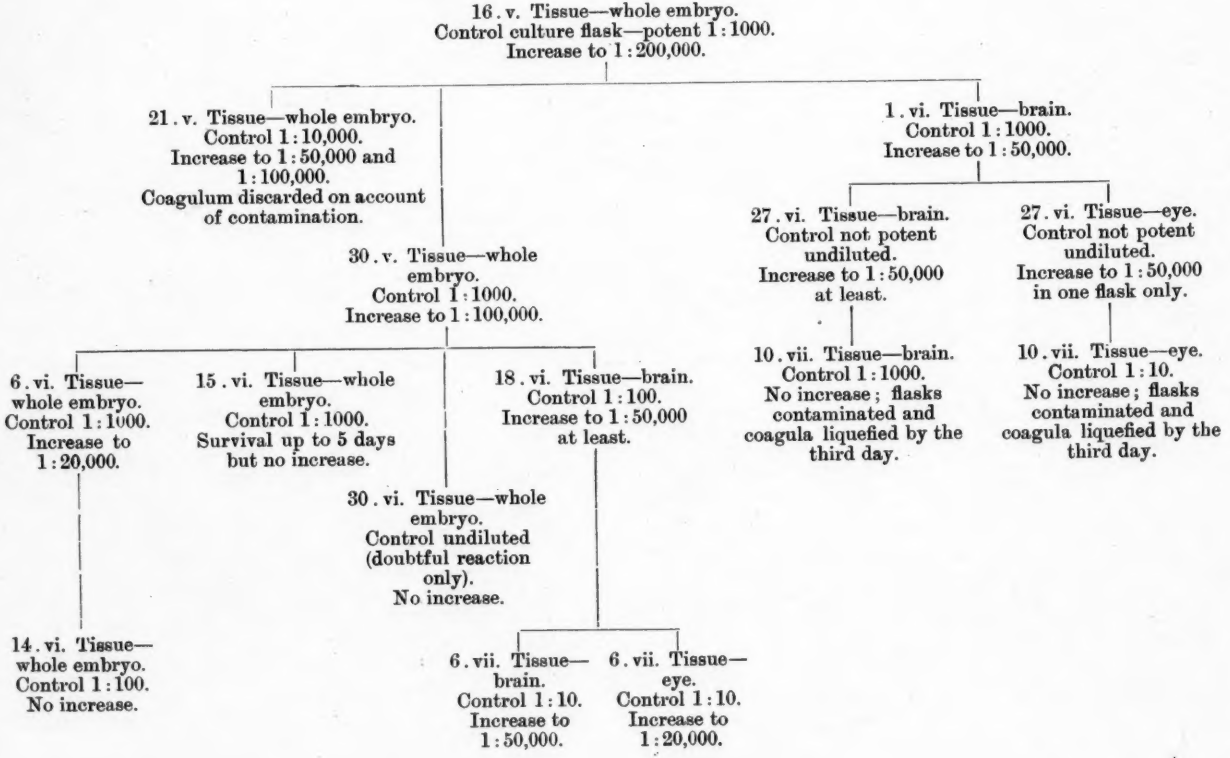
16 . v. Tissue—whole embryo.
Control culture flask—potent 1 : 1000.
Increase to 1 : 200,000.
21 . v. Tissue—whole embryo.
Control 1 : 10,000.
Increase to 1 : 50,000 and 1 : 100,000.
Coagulum discarded on account of contamination.
1 . vi. Tissue—brain.
Control 1 : 1000.
Increase to 1 : 50,000.
30 . v. Tissue—whole embryo.
Control 1 : 1000.
Increase to 1 : 100,000.
27 . vi. Tissue—brain.
Control not potent undiluted.
Increase to 1 : 50,000 at least.
27 . vi. Tissue—eye.
Control not potent undiluted.
Increase to 1 : 50,000 in one flask only.
6 . vi. Tissue—whole embryo.
Control 1 : 1000.
Increase to 1 : 20,000.
15 . vi. Tissue—whole embryo.
Control 1 : 1000.
Survival up to 5 days but no increase.
18 . vi. Tissue—brain.
Control 1 : 100.
Increase to 1 : 50,000 at least.
10 . vii. Tissue—brain.
Control 1 : 1000.
No increase ; flasks contaminated and coagula liquefied by the third day.
10 . vii. Tissue—eye.
Control 1 : 10.
No increase ; flasks contaminated and coagula liquefied by the third day.
30 . vi. Tissue—whole embryo.
Control undiluted (doubtful reaction only).
No increase.
14 . vi. Tissue—whole embryo.
Control 1 : 100.
No increase.
6 . vii. Tissue—brain.
Control 1 : 10.
Increase to 1 : 50,000.
6 . vii. Tissue—eye.
Control 1 : 10.
Increase to 1 : 20,000.

TABLE III.

WHOLE EMBRYO CULTURES.

1. Control potent at 1 : 10.
   After 5 days 1 flask potent at 1 : 10,000 at least
   „ 5 „ 2 flasks „ 1 : 5000
   „ 5 „ 1 flask „ 1 : 1000
   „ 5 „ 1 „ „ 1 : 100
   „ 5 „ 1 „ „ 1 : 10
   „ 8 „ 1 „ „ 1 : 10,000 at least
   „ 11 „ 1 „ „ 1 : 1000

   Summary—
   2 flasks show × 1000 at least.
   1 flask shows × 500.
   2 flasks show × 100.
   1 flask shows × 10.
   1 flask shows no increase.

2. Control potent at 1 : 1000.
   After 5 days 1 flask potent at 1 : 100,000 at least
   „ 5 „ 1 „ „ 1 : 100
   „ 7 „ 2 flasks „ 1 : 100,000 at least
   „ 8 „ 1 flask „ 1 : 50,000
   „ 8 „ 1 „ „ 1 : 10,000
   „ 8 „ 1 „ „ 1 : 1000

   Summary—
   3 flasks show × 100 at least.
   1 flask shows × 50.
   1 flask shows × 10.
   2 flasks show no increase.

3. Control potent at 1 : 1000.
   After 5 days 2 flasks potent at 1 : 20,000
   „ 5 „ 4 „ „ 1 : 1000
   „ 5 „ 3 „ not „ 1 : 10

   Summary—
   2 flasks show × 20.
   7 „ „ no increase.

BRAIN CULTURES.

1. Control not potent undiluted.
   After 3 days 1 flask potent at 1 : 1000
   „ 3 „ 1 „ „ 1 : 10
   „ 5 „ 4 flasks „ 1 : 50,000 at least
   „ 5 „ 1 flask „ 1 : 100,000
   „ 5 „ 1 „ „ 1 : 1000

   Summary—
   4 flasks show × 50,000 at least.
   1 flask shows × 10,000.
   2 flasks show × 1000.
   1 flask shows × 10.

2. Control potent at 1 : 100.
   After 4 days 2 flasks potent at 1 : 50,000 at least
   „ 5 „ 1 flask „ 1 : 50,000 at least
   „ 8 „ 1 „ „ 1 : 10,000
   „ 8 „ 1 „ „ 1 : 5000
   „ 8 „ 1 „ not „ 1 : 10
   „ 10 „ 1 „ „ 1 : 50,000 at least
   „ 10 „ 1 „ not „ 1 : 10

   Summary—
   4 flasks show × 500 at least.
   1 flask shows × 100.
   1 „ „ × 50.
   2 flasks show no increase.

3. Control potent at 1 : 1000.
   After 3 days 1 flask not potent at 1 : 10
   „ 5 „ 2 flasks potent at 1 : 50,000 at least
   „ 6 „ 1 flask „ 1 : 100

   Summary—
   2 flasks show × 50 at least.
   2 „ „ no increase.

4. Control potent undiluted only.
   After 3 days 1 flask potent at 1 : 10
   „ 5 „ 1 „ „ 1 : 50,000 at least
   „ 5 „ 1 „ „ 1 : 1000
   „ 6 „ 1 „ „ 1 : 100
   „ 7 „ 1 „ „ 1 : 10
   „ 7 „ 1 „ not „ 1 : 10

   Summary—
   1 flask shows × 50,000 at least.
   1 „ „ × 1000.
   1 „ „ × 100.
   2 flasks show × 10.
   1 flask shows no increase.

In three of the brain experiments, titrations of some culture flasks were done after three days' incubation only and these all show a small increase of virus compared with that obtained from later titration. The figures for these flasks are included in the " Summary " and, therefore, slightly lower the average yield compared with the whole embryo cultures.

DISCUSSION.

Vaccinia virus has been cultivated successfully by tissue culture methods and carried through four generations in subculture. An increase in virus representing 5 million times the original inoculum has been obtained.

The technique employed is not, however, entirely satisfactory as appears from a study of Tables I and III. When individual cultures in an experiment are titrated after periods of six to sixteen days' incubation it is found that, while increase of virus may be obtained up to twelve days, the maximum increase has occurred about the seventh or eighth day. Wide variation in increase of virus in different flasks is found in any one experiment. It has been shown by the titration of all flasks on the sixth and seventh days that this is not due to length of incubation. It was thought that unequal distribution of tissue in the culture flasks or failure of the tissue to show active proliferation might account for this variation. Repeated experiment has shown quite clearly that neither factor satisfactorily explains this variation since flasks with small amounts of tissue showing no obvious growth have given an increase in virus equal to, or greater than, those with large amounts of tissue in a state of active proliferation. Many experiments have been made in which only an occasional flask showed multiplication of virus though tissue-growth was uniformly good.

It is generally believed by investigators that actively growing tissue is essential to an increase in virus, but it may be that this is not so. Maitland and Maitland (1928) have cultivated vaccinia virus through four generations using minced hen's kidney and hen's serum and Tyrode's solution. So far as they could determine there was no tissue-growth. In their experiments increase in virus took place early reaching its maximum after five days' incubation and it is probable that some portions of the minced kidney-tissue were in a living though not actively growing condition during this period. There is at present insufficient evidence to settle this point and it may be that some factors other than those favouring survival or growth of tissue are necessary to increase of virus.

That multiplication of virus might be favourably influenced by certain tissues has been considered and investigated to some extent. Findlay (1928) cultivated the virus of fowl-pox using minced brain and skin of embryo chicks. He did not state whether these tissues possessed any advantages over whole embryo. As shown in Table III there is good evidence that when brain-tissue is used the multiplication of virus is considerably greater and more uniformly distributed through the culture flasks in an experiment. It is interesting, also, to note in Table II that while the virus had apparently lost its ability to multiply when subcultured in whole embryo, successful multiplication was obtained when it was subcultured at a later date in brain. When eye-tissue was used a very low percentage of flasks showed any increase in virus, but when this increase did occur it was quite equal to that obtained with brain. Skin has not

been investigated. It was thought, for a time, that nerve-tissue included when the eye was removed, and unequal distribution of brain-tissue when whole embryo was used, might account for irregularity in results. Experiments using brain-tissue alone have shown quite clearly by inconstant results that this is not an adequate explanation.

It is possible that our irregular results might be due to strain of virus employed. In some preliminary experiments in which Maitland and Maitland's technique was followed, using a dermal virus as seed, increase has not so far been obtained and it occurred to us that choice of strain might explain our failure to confirm their results. A supply of neuro-vaccine, adapted to rabbit's testicle, was obtained through the kindness of Dr. C. H. Andrewes, and further culture experiments made with it using the Maitlands' method. While there is not yet sufficient evidence to form a definite conclusion the results have been encouraging and experiments are being carried on with this strain by both Maitland and Maitland and Carrol and Rivers technique. In a short series of experiments using testicular vaccine with chick embryo brain a comparatively uniform increase in virus has been obtained. It is difficult to explain why this strain of virus should behave in a different way to dermal virus, but it may be that the testicular extract contained in the virus fluid exerts an enhancing influence as pointed out by Reynals (1928).

That the virus has not changed its character through cultivation is apparent when lesions produced by the virus before and after cultivation are compared by scarification and intradermal inoculation. Moreover, culture virus is neutralized by the serum of a vaccine-immune rabbit.

As shown in Table I a large quantity of culture virus has been obtained using a Roux flask. It was hoped that this would supply a stock of virus for subculture experiments. It is disappointing to find that this culture virus is inactivated very rapidly at cold room temperature. Since dermal virus may be kept for some months in contact with the tissue, but is inactivated rapidly in the diffusate when removed therefrom, attempts have been made to simulate the conditions by leaving the virus in contact with the ground culture material. Neither this, nor the addition of glycerin has proved of value and further work is being undertaken to discover, if possible, a satisfactory method of preserving culture virus.

CONCLUSIONS.

1. Vaccinia virus has been cultivated using tissue-culture methods and carried through four generations in subculture.

2. Multiplication of dermal virus occurs irregularly in that only occasional flasks in an experiment show increase though conditions are apparently similar in all.

3. Our experiments suggest that active growth of tissue may not be essential to increase of virus. When present it is not an indication that increase in virus will occur.

4. There is evidence that brain-tissue is more suitable than whole embryo for cultivation of vaccinia virus. Its use does not, however, result in a regular increase in virus.

5. When testicular vaccine is used a comparatively regular increase in virus has been obtained.

6. Typical vaccinial lesions are produced by culture virus by scarification and intradermal inoculation.

7. Culture virus is neutralized by vaccinia-immune rabbit-serum.

8. Unlike calf-lymph, virus obtained by culture deteriorates rapidly at cold room temperature.

REFERENCES.

CRACIUM, E. C., AND OPPENHEIMER, E. H.—(1926) *J. Exp. Med.*, **43**, 815.
CARREL, A., AND RIVERS, T. M.—(1927) *C. R. Soc. Biol.*, Paris, **96**, 848.
FINDLAY, M.—(1928) *Brit. J. Exp. Path.*, **9**, 28.
HAAGEN, E.—(1928) *Zbl. Bakt.*, **109**, 31.
LEDINGHAM, J. C. G., AND MCCLEAN, D.—(1928) *Brit. J. Exp. Path.*, **9**, 216.
MAITLAND, H. B., AND MAITLAND, M. C.—(1928) *Lancet*, ii, 596.
PASCHEN, E.—(1906) *Zbl. Bakt.*, I. Abt. Ref., **41**, 637.
PARKER, F., JUN.—(1923–24) *J. Med. Res.*, **44**, 645.
PARKER, F., JUN., AND NYE, R. N.—(1925) *Amer. J. Path.*, **1**, 325.
REYNALS, F. D.—(1928) *C. R. Soc. Biol.*, Paris, **99**, 6.

A QUANTITATIVE STUDY OF THE EFFECT OF ULTRA-VIOLET RAYS UPON THE BACTERIOPHAGE.

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PART I.—A COMPARISON BETWEEN THE LETHAL EFFECTS OF ULTRA-VIOLET RAYS UPON THE BACTERIOPHAGE, *B. COLI* AND SOME FERMENTS AND ANTIBODIES.

THE bacteriophage is particularly suitable for an investigation into the effect of ultra-violet rays upon a filter-passing agent. It is active in very high titre, and can be used in a dilution which is practically as transparent as distilled water to the bactericidal rays. Its presence can be easily and rapidly detected, and a quantitative estimation can be made with much greater accuracy than is possible when animal inoculation has to be employed.

The destructive effect of ultra-violet rays (of wave-length shorter than 3000 Å) upon bacteria, several forms of ultra-microscopic virus, ferments and certain antibodies has been demonstrated by various workers.

Although a considerable amount of work has been done upon the bactericidal efficiency of different parts of the spectrum, there appear to be

few publications in which a comparison is made between the lengths of exposure necessary to destroy or inactivate the various classes of body, bacteria, ferments, etc., mentioned above.

One of the difficulties of making such a comparison arises from the fact that there is no unit of measurement of the bactericidal output of a mercury-vapour lamp or other source of ultra-violet rays. There has, therefore, been no method by which different workers can compare their results in a quantitative manner. One of us (S. L. B.) and Peacock (1926) used as a standard of comparison the lethal effect upon *B. coli*. In the present work we have employed the same *B. coli* control, and we have been at considerable trouble to standardize our technique and to work out precise curves showing the course of destruction of this organism with varying exposures to ultra-violet rays. There is no doubt that such a bacterial control coupled with a satisfactory technique gives a precision to the bactericidal dosage which could not be achieved by any of the present chemical or photographic methods of measurement.

In the paper mentioned above it was shown that the infective agent in Berkefeld filtrates of the Rous sarcoma was less susceptible to ultra-violet radiation than are the common pathogenic bacteria; with five times the lethal dose for *B. coli* the infective agent was still active though attenuated. Further work has shown that with about eight lethal *coli* doses the filtrate is usually rendered completely inactive. In the present work we have examined carefully the action of ultra-violet rays on one particular strain of Shiga bacteriophage, and also upon a strain of staphylococcus bacteriophage, comparing the effect quantitatively with that observed upon a strain of *B. coli*—the same strain as was used in the previous experiments on the Rous agent. As a comparison we have also estimated the effects of varying lengths of exposure upon diastase, trypsin, hæmolytic amboceptor, complement and lysozyme from human tears.

Several writers have demonstrated that the bacteriophage is destroyed by ultra-violet rays, but up to the present very few quantitative results have been available.

Applemans (1922) added one drop of an active bacteriophage to 3 c.c. of normal saline and exposed this in a quartz tube to ultra-violet rays for 10 minutes; a similar dilution was exposed for the same time in a glass tube. That in the quartz tube was found to be inactive, while that in the glass tube was active in a dilution of 10^{-8} to 10^{-9}.

Zoeller (1923) performed a similar experiment in which the irradiation lasted 15 minutes; he found that the bacteriophage was destroyed, as was also Shiga's bacillus treated in the same way.

The experiments of these workers show clearly that the bacteriophage is destroyed by ultra-violet radiation, but do not give any indication as to whether the lethal dose is greater or less than that required for bacteria.

Gildmeister (1922) and McKinley, Fisher and Holden (1926) exposed bacteriophage in bouillon for 45 and 40 minutes respectively to the rays of the mercury-vapour lamp. The first worker states that the bacteriophage was inactivated, while *B. coli* treated in the same manner was sometimes eliminated and sometimes survived. McKinley and his co-workers

produced sometimes inactivation, and at other times attenuation of the bacteriophage, but they state that no apparent effect was produced on the strain of *B. coli* exposed in the same manner. The technique employed by both these workers in the above experiments appears far from satisfactory and it is impossible to attach any quantitative value to their results.

It is well known that ordinary beef bouillon is very opaque to ultra-violet rays, and the statement of the latter workers that "no apparent effect" was produced on a strain of *B. coli* with 40 minutes' exposure indicates that only a very small percentage of the bactericidal range of the spectrum could have escaped absorption. Conditions of this sort invite errors from various sources.

Fisher and McKinley (1927) published another series of experiments upon the action of ultra-violet rays on a bacteriophage. They found a logarithmic rate of destruction, but no mention is made of lethal effects on *B. coli* or other bacteria under the same conditions of exposure.

Gerretsen, Gryns, Sach and Söhngen (1924) studied the effect of ultra-violet rays upon a bacteriophage found, associated with the nitrifying bacteria, in the root-nodules of certain leguminous plants.

They adopted two methods of exposure:

(1) Agar plates were stroked with bacteriophage suspension and then exposed for 5, 15 and 30 minutes at 30 cm. from a mercury-vapour lamp.

The plates were then inoculated with a culture of the bacterium. No bacterial growth took place on the areas inoculated with the bacteriophage, proving that the latter was still active.

(2) Fluid containing the bacteriophage was exposed in a thin layer, and it was found that it required 2½ hours to produce inactivation. The bacterium was destroyed under the same conditions in 15 minutes.

These results show that this particular bacteriophage is more resistant to ultra-violet radiation than is the associated bacterium. It is not clear, however, that the ratio of 10 : 1 shown by the exposure times represents the relative susceptibilities of the two agents. If the bacteriophage were in much greater concentration than the bacillus, as is probable, this numerical factor would, by itself, increase somewhat the time necessary for sterilization. This question is discussed more fully below.

Technique of Exposure.

Throughout our experiments the bacterium or other agent to be irradiated has been suspended in normal saline. The fluid to be treated is exposed in an open dish.

In the present investigation a K.B.B. atmospheric mercury vapour lamp was used; this was run at 225 volts, using 2·2 amps. The lamp was also used for photographic purposes and was arranged to give a horizontal beam. A mirror consisting of a sheet of quartz backed by a mercury amalgam on tin was, therefore, used to reflect the rays vertically downwards onto the surface of the fluid to be treated.

This arrangement led, of course, to some general loss at the reflecting surface of the mirror but there was a good transmission of the short end of

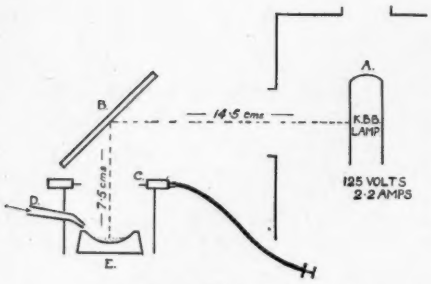

FIG. 1.

FIG. 1.—Arrangement of apparatus for irradiating bacterial suspensions, etc. A. Mercury vapour lamp enclosed in metal shield. B. Mirror, quartz plate backed by mercury amalgam on tin. C. Shutter with cable release for accurate control of shorter exposures. D. Stirring device, tube delivering a fine jet of sterile air on to the surface of the liquid. E. Open glass dish containing fluid to be irradiated. The standard volume of fluid was 0·6 c.c., which gave a layer with a maximum depth of 2·5 mm. in the centre.

the spectrum, and on examining the reflected beam with one of Beck's small ultraviolet spectroscopes, the collection of faint lines giving a rather diffuse luminosity at the short end of the spectrum could be seen as far as 2100 Å.

The general arrangement of the apparatus is shown in Fig. 1. The fluid under irradiation was outside the lamp-box, manipulation was easy and there was no fear of overheating. The same open dish was used throughout. Samples of 0·6 c.c. were irradiated; this volume gave a thickness of 2·5 mm. at the deepest part of the fluid. The fluid was kept in continuous motion during irradiation by a fine jet of sterile air.

A photographic shutter controlled by a cable release was placed above the dish and enabled exposures of a few seconds to be made with an accuracy within about a fifth of a second.

Use of B. coli *Control.*

Throughout our experiments each series of exposures is controlled by a parallel series in which *B. coli* is suspended in the fluid under treatment—bacteriophage suspension, diluted ferment, etc. By this procedure the amount of effective bactericidal rays reaching particles suspended in the particular fluid under examination can be controlled. In all experiments upon the effect of ultra-violet rays, it is obviously advisable to ensure that the fluid to be treated is as transparent as possible to the wave-lengths between 3000 and 2000 Å. In the case of bacteria or bacteriophage it is easy to arrange a high dilution in saline. Although undiluted meat broth is too opaque to the rays for use in experiments on ultra-violet rays, it may be added to normal saline in the proportion of 1 to 1000 without producing any appreciable increase in opacity to the bactericidal wave-lengths in a layer less than 1 cm. thick. In some cases it is not possible to avoid a moderate degree of opacity to ultra-violet rays in the fluid to be treated.

This was the case with the trypsin solution used in the present series of experiments. It was necessary to obtain a series showing a progressive diminution in tryptic activity, and it was hardly practicable to use an initial dilution beyond 1 in 200. This dilution was appreciably opaque to the bactericidal wave-lengths, and on working out a series of exposures upon *B. coli* suspended in the solution under the conditions of experiment it was found that the time taken to destroy 90% of the bacilli was 33 seconds. Now

with a dilution of *B. coli* in saline (containing not more than 1/1000 bouillon) the time taken to produce a 90% destruction was about 14 seconds; hence it appears that somewhat less than 50% of the bactericidal rays penetrated the solution. It is obvious that a correction must be made for this absorption, since only the most superficial part of the trypsin solution is getting the full dose; this can easily be done by reckoning 33 seconds of exposure as equal to 14 seconds under standard conditions in saline.

This method of control is simple, and can be carried out with any fluid which has no lethal action on *B. coli*; all our results on ferments or other fluids are corrected and standardized in this manner.

Preparation of B. coli suspension.—Bacterial suspensions to be irradiated should conform to certain essential conditions, and if the lethal effect of ultra-violet rays upon a bacterium is to be used as a method of estimating bactericidal power, it is obviously important to standardize, as far as possible, the suspensions used.

The essential conditions of such a bacterial suspension are: (1) It should contain a reasonably large number of organisms, but not sufficient to produce an appreciable opacity to the bactericidal rays. We have found concentrations between 100,000 and 500,000 bacilli per c.c. to be satisfactory.

(2) The suspension should consist, as far as possible, of isolated organisms, and should be free from microscopic clumps. There is no doubt that even a moderate amount of clumping may considerably prolong the bactericidal time, and it appears very probable that the variations in susceptibility which have been found in cultures of different ages are largely dependent upon the degree of aggregation of the organisms.

We have tried various methods of producing the bacterial suspension and have finally adopted the following as the standard procedure: (1) Inoculate an agar slope with *B. coli* and incubate at 37°C. overnight. (2) Remove several loopfuls of the young growth and emulsify carefully by rubbing down in a drop of saline, then add normal saline and adjust to a standard opacity. (3) With this suspension inoculate a tube of ordinary broth so that it contains about 10 million organisms per c.c. (4) Warm to 37°C. and incubate at this temperature for 4 hours. In this time (in the case of our strain of *B. coli*) the organisms increase to 200–300 million per c.c.

The bacilli are present singly or at most in pairs; there are no microscopic clumps; 90% of the organisms are less than 4 hours old. This standard culture may now be diluted 1 in 1000 in saline giving a suspension for irradiation with 200–300 thousand *B. coli* per c.c. and containing a negligible amount of broth.

It is important to use a fresh slope culture for inoculating the fluid medium; we have found that older cultures show an initial lag in growth and do not increase much in the first few hours.

Methods of Estimating the Lethal Effect of Irradiation.

If a susceptible agent such as a bacterium or a ferment is acted upon by ultra-violet rays it undergoes a continuous destruction until the extinction point is reached, that is, until the test employed to detect the presence of the agent gives a negative result.

The results of such an experiment on B. *coli* were charted by plotting the numbers of survivors against the exposure times and the left-hand curve (Fig. 2) was obtained; when the logarithms of the numbers of survivors were plotted as ordinates the right-hand curve (Fig. 2) was obtained. We have plotted in this way the results of over a dozen such experiments on *B. coli*, and with logarithmic ordinates the same type of convex curve has always been obtained. Such a curve showing a continuous increase in the rate of destruction would be produced by an agent having a cumulative action.

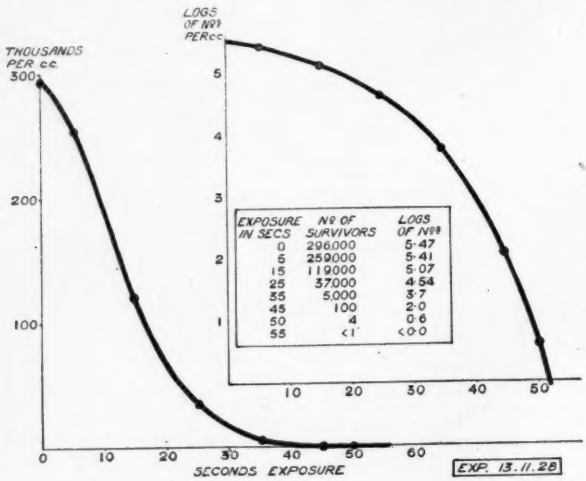

FIG. 2.

FIG. 2.—Chart showing the effect of irradiation on *B. coli*. In the left-hand curve the numbers per c.c. surviving after various lengths of exposure are plotted as ordinates. In the right-hand curve the logarithms of these numbers are plotted. The sign <1 in the table indicates sterility, i.e. less than 1 organism in a 1·0 c.c. sample. The latter curve becomes steadily steeper as the base line is approached, differing from that found in the case of the destruction of bacteria by antiseptics.

Yule (1910) discussed survival-curves produced by the cumulative action of a noxious agent on a population, but, as far as we are aware, no previous experimental work has demonstrated such a curve. Those who have worked on the rate of destruction of bacteria by antiseptics show curves which are practically straight in the middle and do not become steeper at the end, but flatten out so that the time for complete sterilization is much prolonged. This effect appears to be due to a prolonged survival of a few very resistant organisms. It is possible that the range of variation among individual bacteria in their resistance to ultra-violet rays is more limited, and that we therefore get the pure cumulative effect which is masked in the case of other lethal agents. We propose to consider these questions more fully in a later communication.

Note.—A few points in connection with the use of the logarithms, rather than the numbers in the construction of the above curves, may be acceptable to those who have not had occasion to consider the matter.

The mode of destruction of a bacterial population under the influence of any strong lethal agent is such that the number killed in any short period of time is of the order of the number alive at the beginning of that period. Thus, if a given population is reduced to a tenth in, say, x seconds, the fall in the next x seconds is likely again to be in the neighbourhood of 90% of the number *surviving* at the end of the first x seconds, so that the actual number of organisms dying in the second x seconds will be very small compared with the number dying in the first similar period.

To demonstrate this mode of destruction graphically, the survivors at any instant may be charted against the time on an ordinary scale. Very incomplete information, however, can be obtained from such a curve. The bulk of the population will have been destroyed almost immediately and, whether the rate of destruction is increasing, constant or diminishing, the behaviour of the remainder can be represented only by a slightly curved line very near the base-line and constantly approaching it (*vide* left-hand curve, Fig. 2). For this reason, logarithms of the numbers are usually plotted against time. As the ordinate units in a curve of logarithms rise in geometrical progression, the process of destruction can be accurately followed right up to the point of extinction. Extinction occurs when the number of survivors is less than one, the logarithm of which is zero—*i.e.* where the curve meets the base-line.

The curve of the logarithms may be convex upwards, straight or concave upwards. A straight line signifies that the death-rate is constant—in other words, in every unit of time the same percentage of the survivors is being destroyed. A convex curve signifies an acceleration of the death-rate, *i.e.* a greater and greater *percentage* is being killed every moment—a behaviour we would expect from a noxious agent with cumulative action. A concave curve, on the other hand, would indicate a retardation such as would be produced by a few organisms resisting the action of the lethal agent to a much greater extent than the bulk of the population.

The lethal effect of ultra-violet rays upon bacteria is often estimated by the length of exposure required to sterilize a fluid suspension or an inoculated agar surface. This method is less laborious than that of estimating the proportions destroyed by varying lengths of exposure, and as long as the two types of organism which are being compared are present in approximately the same initial numbers per c.c., a fairly accurate comparison may be made. In the case, however, where the organisms or agents exist in very different concentrations, there may be a serious fallacy in using the extinction points as a standard of comparison. The following example will make this matter clear: A suspension of *B. coli* containing 1 million organisms per c.c. was prepared (= suspension A). This was diluted 1/100 and again 1/100, giving suspension B (10^4 per c.c.) and suspension C (10^2 per c.c.). The diluent in each case was a portion of suspension A which had been raised to boiling-point in order to sterilize it. There were now three suspensions containing the same number of bacillary bodies each, but containing respectively 10^6, 10^4 and 10^2 living *B. coli*. Samples of each of these suspensions were irradiated for varying times. A suitable dilution was mixed with agar, plated, and colony counts were made. The curves shown in Fig. 3 indicate the logarithms of the numbers surviving. The sterility point is reached in each case when the curve cuts the base line; it occurred with sample A after 50 seconds, with sample B after 40 seconds and with sample C after 30 seconds.

An examination of the curves shows that the points on B and C maintain very nearly a distance of 2 and 4 divisions respectively on the log. scale below the corresponding point on curve A. In other words, by merely shifting the

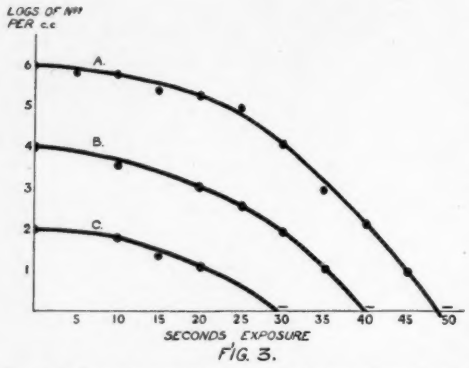

FIG. 3.

FIG. 3.—Experiment showing the effects of irradiating suspensions of *B. coli* of different concentrations. Curve A represents a suspension containing 1 million (10^6) *B. coli* per c.c.; curve B a suspension containing 10 thousand (10^4); and curve C a suspension containing 1 hundred (10^2) organisms per c.c. The point where each curve cuts the base line gives the time required to sterilize the sample in each case. The – signs indicate that samples were sterile.

base line up either 2 or 4 divisions curves B and C can be formed from curve A. This is what would be expected from purely numerical considerations. It illustrates, however, in an actual experiment the fact that the time taken to sterilize cannot be used as a comparison of ultra-violet susceptibilities unless the concentrations of the agents under test are of approximately the same order.

Such a source of fallacy might easily arise when comparing the bacteriophage with a bacterium, since the former may exist in very high concentrations (a dilution of 10^{-12} may be required to reach an end-point). As mentioned above, this factor probably operated to prolong somewhat the exposure necessary to destroy the bacteriophage in the experiment of Gerretson and his co-workers.

Preparation of the Bacteriophage.

The bacteriophage chiefly employed in these experiments had been recovered from the fæces of a normal guinea-pig four months previously and, in order to exalt its virulence, it had been passed seventeen times through a susceptible culture of *B. dysenteriæ* Shiga. At the time of starting the present experiments this bacteriophage was active in a dilution of one in a hundred thousand million (10^{-11}). The bacteriophage was stored in 1 c.c. ampoules; this stock fluid showed no diminution in titre during the period of the experiments. The stock bacteriophage was diluted to 1/10,000 with saline before irradiation ($= B/10^4$). This dilution reduced the amount of bouillon present to a negligible quantity, and also produced a bacteriophage concentration of about the same order as that of the *B. coli* control suspensions. Samples of this diluted bacteriophage were irradiated for varying times as described above; the amount surviving in each sample was then estimated by—

(*a*) Serial dilutions in broth;
(*b*) Plaque counts.

(a) *Estimation of bacteriophage titre by serial dilutions.*—A series of broth tubes each containing 4·5 c.c. was prepared; 0·5 c.c. of the bacteriophage fluid ($B/10^4$) was added to the first tube and the same quantity was then transferred from tube to tube, giving a series of decimal dilutions. To each of these dilutions was then added a sufficient quantity of a fresh culture of *B. dysenteriæ* Shiga to give about 100 million bacilli per c.c.; the tubes were

incubated for 24 hours and agar slopes then inoculated. Next day these were examined for evidence of lysis and the broth tubes for signs of clearing.

Since it was possible that the bacteriophage might still be present in small quantity in the apparently negative broth-tubes, these were subcultured onto agar on two successive days and examined for the presence of lysis.

The contents of the two lowest dilution tubes which still failed to show the presence of bacteriophage by this means were now filtered first through alkaline kieselguhr and then through a Chamberland L2 candle. This filtrate was diluted 1/4 with additional broth and inoculated lightly with the Shiga bacillus; this inoculated broth was, after 24 hours, subcultured onto agar. The absence of lysis on this subculture was taken as a final negative as regards the presence of bacteriophage.

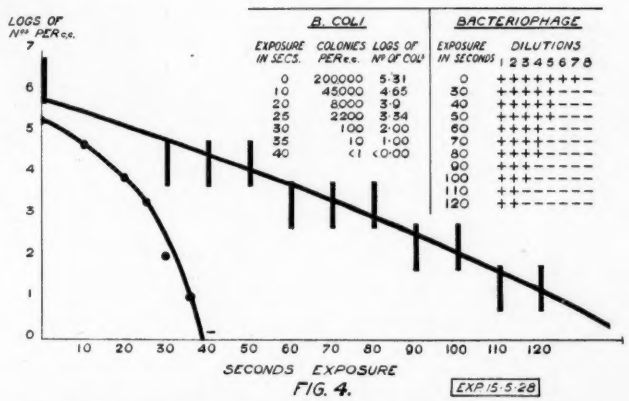

FIG. 4.—Chart showing survival curves of *B. coli* and Shiga-bacteriophage under irradiation. The logarithms of the numbers per c.c. (or concentration) are plotted as ordinates. The left-hand curve shows the effect on *B. coli*; it is a similar curve to those plotted in figs. 2 and 3. The right-hand curve is drawn to cut the series of vertical lines which represent the approximate concentrations of bacteriophage at various points, as estimated from the results shown in the table. The sign <1 in the left-hand table is used as in fig. 2, and the − sign at 40 seconds indicates a sterile sample. The dilutions shown in the table are $1/10$, $1/10^2$ . . . $1/10^8$.

After exposure to a certain dose of irradiation each sample of bacteriophage ($B/10^4$) was diluted down through 8 tubes by the above method and the last positive tube was taken as an indication of the proportion surviving. The untreated fluid ($B/10^4$) gave a positive in the 7th tube, showing a titre of 10^7 (that is of 10^{11} in the original undiluted bacteriophage). The titre of the fluid after irradiation showed a steady diminution as the length of the exposure increased. One set of results of serial dilutions of irradiated bacteriophage is shown in Fig. 4.

When estimating the titre of a substance by serial dilutions it is obviously unjustifiable to assign an exact figure (although this is frequently done); one can only conclude that the concentration lies between certain limits determined

by the interval between the adjacent tubes of the series. In our series we have indicated these limits by vertical lines, and as smooth a curve as possible is drawn so as to cut all the lines.

(b) *Estimation of the bacteriophage titre by plaque counts.*—There is no doubt that it is much more difficult to obtain satisfactory plates for counting in the case of the bacteriophage than in the case of bacteria, but allowing for these difficulties we have found that with our Shiga-bacteriophage the number of plaques diminish approximately in proportion to the dilution of the inoculum.

After several preliminary experiments the following procedure was employed to estimate the titre of a series of samples of bacteriophage which had received varying exposures to ultra-violet rays: A set of dilutions similar to that used in the previous series was made in broth containing 5% gelatin. From previous experiments a rough estimate had been made of the number of plaques to be expected from the tubes in the neighbourhood of the end-point, and from each series a tube estimated to yield 100–200 plaques per c.c. was selected. This tube was inoculated with *B. dysenteriæ* Shiga to the extent of about 300 million per c.c.; six samples of 0·2 c.c. were removed, and each was spread as evenly as possible over the surface of an agar plate. It was found that the addition of 5% gelatin to the broth facilitated an even spreading of the inoculum on the plates and did not interfere with plaque-formation (Fig. 6).

Table I shows the result of this experiment. The bacteriophage had been diluted 1/10,000 before irradiation. The figures in the fourth column give the number of bacteriophage particles per $\frac{1}{2}$ c.c. (the standard volume used in the broth dilutions) as estimated from the plaque counts. This is seen to be 100,000 in the untreated fluid, and the numbers diminish with each increase of exposure until with 90 seconds' exposure only 12 plaques remain; with 120 seconds' exposure both plates and broth tubes were negative.

Note.—This series of plaque counts does not form a smooth curve: when expressed graphically there is a sudden drop at first; after this the curve is smooth. We have not repeated this type of experiment sufficiently often to say whether there is any significance in the preliminary fall seen here. Apart from this irregularity the plaque counts are in fairly good agreement with the results in broth.

TABLE I.—*Showing Effect of Irradiation upon the Production of Plaques.*

Duration of exposure (seconds).	Dilution before plating.	Number of plaques obtained in the various plates, each inoculated with 0·2 c.c.						Average number of bacteriophage units in 0·5 c.c.
0	$1/10^3$	30	38	54	33	45	47	100,000
15	$1/10^2$	18	13	16	14	12	7	3,300
30	$1/10^2$	6	7	3	7	4	11	1,600
60	1/2	46	43	36	60	37	32	212
90	1/1	6	4	...	...	...	...	12
120	Active bacteriophage could not be recovered							

Methods Used for Estimating the Effect of Irradiation on the Ferments and Antibodies Tested.

Trypsin.—Allen & Hanbury's *Liquor trypsinæ* diluted 1/200 was exposed under standard conditions as described.

The tryptic activity was estimated by the liquefaction of gelatin at pH 8. Various dilutions of trypsin were mixed with equal parts of the standard gelatin solution giving a range of titres 1/400, 1/800 . . . 1/25,600. On the basis of this titration it was found that the titre fell to 1/10 with an exposure equivalent to 300 seconds, while *B. coli* dropped to this value in 15 seconds.

Complement.—The titre was estimated in the usual manner with sensitized red cells. The value fell to about 1/3 with an exposure equivalent to 180 seconds.

Hæmolytic amboceptor.—Rabit serum hæmolytic for sheep-cells was diluted 1/500 in saline. Samples of this were irradiated for varying times and the titre was estimated in the usual manner with red cells and complement. The value fell to 50% after 360 seconds' irradiation and to 10% after 720 seconds.

Diastase.—Taka diastase in a dilution of 1/1000 in saline was irradiated for varying periods. Serial dilutions were made with each sample, and an equal volume of 1/1000 soluble starch buffered at pH 6·1 with phosphate was added. After incubation the digestion was estimated colorimetrically by the addition of iodine solution.

Time of exposure.	% remaining.
0 minutes	100
10 ,, ...	50
20 ,, ...	20
30 ,, ...	10

Lysozyme from human tears.—A dilution of 1/200 of human tears was irradiated for varying periods. A series of further dilutions was prepared from each sample and tested against a suspension of *M. lysodeikticus*. The result is given in the table below:

Exposures in minutes.	Dilution.				
	1/200	1/800	1/3200	1/12,800	1/51,200
0 . . .	...	++	+	±	—
4 . . .	...	++	+	±	—
16 . . .	...	+	±	—	—
32 . . .	+	±	—	—	—

++ = complete; + = partial; ± = trace of lysis.

From this experiment it appears that an exposure of 16 minutes reduces the titre to 1/4, and 32 minutes' exposure reduces it to about 1/16.

Complement reduced to about 30% in 180 seconds. A staphylo-bacteriophage, tested in one rough experiment only, was somewhat less resistant to irradiation than our Shiga bacteriophage.

TABLE II.—*Summary of Experiments.*

		Number of seconds required to reduce numbers or titre to—				
		10%	1%	0·1%	0·01%	0·001%
A. (15.5.28)	Bacteriophage . .	25	50	70	100	125
	B. coli control . .	15	25	30	34	37
B. (8.5.28)	Bacteriophage . .	30	45	60	90	<120
	B. coli control . .	18	22	27	31	35
C. (18.12.28)	Bacteriophage (plaques)	10?	42	72	93	<120
	B. coli control . .	25	36	42	47	52
D. (29.5.28) (screened)	Bacteriophage . .	112	210	312	420	540
	B. coli control . .	90	114	138	144	162
E.	Trypsin . . .	300	...	...	...	...
	Hæmolytic amboceptor.	720	...	...	...	...
	Diastase . . .	1800	...	...	...	...
	Lysozyme . . .	1800	...	...	...	...
	B. coli control . .	15	...	...	...	...

Discussion of Results.

Table II gives a summary of the experimental results, and Fig. 5 gives a good idea of the destructive effect of irradiation upon the various bodies examined.

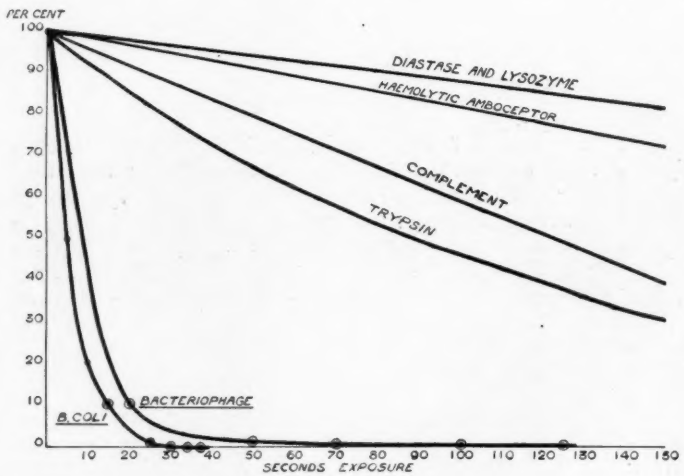

FIG. 5.

FIG. 5.—Chart showing a comparison between the effects of irradiation upon *B. coli*, the Shiga-bacteriophage and various ferments, etc. The curves indicate the percentage remaining after various lengths of exposure. The upper four curves represent in each case only a small portion of the entire curves, the figures of which are given in the text.

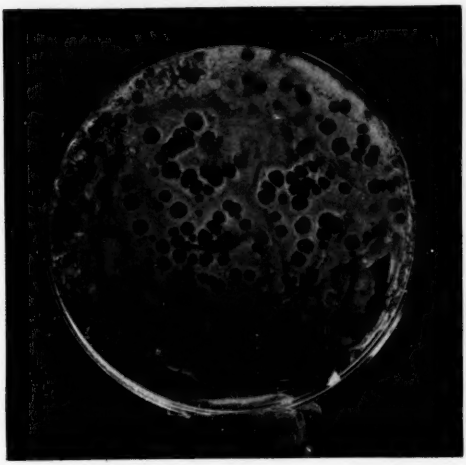

FIG. 6.—Photograph of an agar-plate showing lytic areas produced by bacteriophage on a culture of *B. dysenteriæ* Shiga. It shows two types of plaque, large rounded areas and small pin-head areas. The plaques are too numerous for accurate counting as many of the larger ones are confluent.

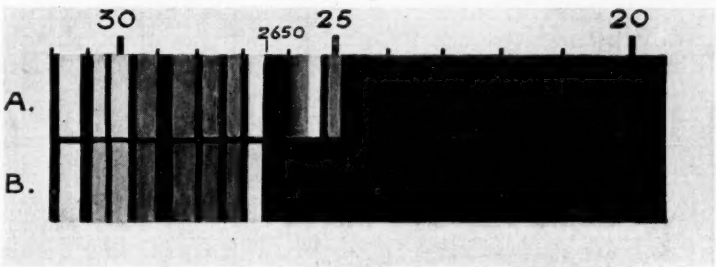

FIG. 7.—Drawing of portion of the mercury vapour spectrum as seen with Beck's small ultra-violet spectroscope. The scale indicates the wave-lengths from 3200Å to just below 2000Å. The spectrum A is for unscreened rays. The spectrum B shows the effect of interposing a quartz cell containing 0·5 % cobalt chloride in absolute alcohol, the thickness of the solution being 1 cm. It is seen that there is a sharp cut-out of the shorter wave-lengths at 2650Å.

The effect of a destructive agent upon different types of organism, ferment, etc., may differ not only quantitatively but qualitatively. A comparison between the susceptibilities to irradiation of different types of organism cannot be expressed as a single numerical ratio if the survival curves are of different forms. In one case, for instance, (*a*), the bulk of the population may be destroyed very quickly but a certain small proportion may survive a much longer time, while in another case, (*b*), there may be a slower rate of killing at first which may be compensated for by a more rapid destruction later, the net result being that both communities become extinct in about the same time. A comparison between the numbers of survivors in the communities (*a*) and (*b*) at different periods during the course of destruction will not show constant ratios. This result is seen when the exposure times for *B. coli* and the bacteriophage are compared as in Table II.

When considering the effect of irradiation on a bacterial population one is concerned with the effect on the *bulk of the population*; the few survivors in the latter part of the period of exposure, although of importance from the point of view of sterilization, are of very little significance as an indication of the resistance of an average member of the community. Hence when comparing the susceptibilities of *B. coli* and the bacteriophage in Fig. 4, the nearly horizontal extensions of the curves are relatively unimportant.

In spite of possible dissimilarities in the details of the process of destruction, one can give figures indicating roughly the relative susceptibilities to irradiation of the various agents so far examined. Taking the exposure necessary to destroy an average *B. coli* as 1, that required for—

Shiga-bacteriophage = about 2.
Staphylo-bacteriophage = 1 to 2.
Trypsin and complement = 20 to 30.
Hæmolytic amboceptor = about 50.
Diastase and lysozyme = about 120.

PART II.—THE EFFECTS OF DIFFERENT PORTIONS OF THE SPECTRUM UPON THE BACTERIOPHAGE AND UPON *B. COLI*.

In the paper by one of us (S.L.B.) and Peacock (1926) the view was expressed that the marked resistance of the infective agent of the Rous sarcoma to ultra-violet rays might be due to its small size. The results of the present work on the bacteriophage appear to negative this suggestion. Judging from filtration experiments the bacteriophage particle must be considerably smaller than that of the Rous agent, yet the bacteriophage is more susceptible to irradiation than is the infective agent of the fowl sarcoma.

It appeared possible that a particle of a size small in comparison with the wave-length of the incident rays might escape part of the destructive effect. If this were so one would expect that the longer wave-lengths of the bactericidal range would be relatively less effective on such a small particle than on an ordinary bacterium.

In order to test this possibility we have screened off the shorter end of the bactericidal range of the spectrum, and have examined the effect of the remainder upon *B. coli* and upon the bacteriophage.

First of all in order to satisfy ourselves that the bacteriophage was not susceptible to the non-bactericidal portion of the spectrum from 3000–4000° Å, we exposed a sample under the conditions described earlier, but covered the dish (Fig. 1, E) with a piece of glass. An exposure of 1¾ hours produced no diminution in the titre, whereas without the glass cover an exposure of about 50 seconds destroyed 99% of the activity. The next step was to find a suitable screen which would cut out a portion of the bactericidal range as sharply as possible, leaving the remainder little affected. We are indebted to Dr. R. B. Bourdillon for suggesting the use of a solution of cobalt chloride in absolute alcohol. He kindly supplied us with the following figures, indicating, approximately, the percentage of radiation transmitted at various wave-lengths with a screen of 0·5% alcoholic cobalt chloride solution used in a thickness of 1 cm.:

Wave-length:	4000	3342	2967	2900	2805	2760	2700	2654	2537	2482
% transmitted:	87	89	87	86	84	80	77	63	2·9	1·8

It can be seen from these figures that there is a sharp cut-out of all but a trace of the spectrum for wave-lengths shorter than 2650° Å.

In our experiments we used a solution of 0·5% cobalt chloride in alcohol contained in a quartz cell of 1 cm. thickness internally. The mercury vapour spectrum as seen on the fluorescent scale of one of Beck's small ultra-violet spectroscopes is shown in Fig. 7 (A). The appearance when the cobalt chloride screen was interposed is shown in spectrum (B).

Using this filter *B. coli* and Shiga-bacteriophage were irradiated for varying times by the technique already described in Part I. The tables III and IV give the results of these experiments:

TABLE III.—*Results of Irradiation of the Bacteriophage through the Cobalt Chloride Screen.*

Duration of exposure in minutes.	Dilutions. 1.	2.	3.	4.	5.	6.	7.	8.
0	+	+	+	+	+	+	+	−
1	+	+	+	+	+	+	−	−
2	+	+	+	+	+	−	−	−
3	+	+	+	+	+	−	−	−
4	+	+	+	+	+	−	−	−
5	+	+	+	+	−	−	−	−
6	+	+	+	+	−	−	−	−
7	+	+	?	−	−	−	−	−
8	+	+	−	−	−	−	−	−
9	+	−	−	−	−	−	−	−
10	+	−	−	−	−	−	−	−

The dilutions are the same as those shown in Fig. 4; + = bacteriophage recovered.

TABLE IV.—*Results of Irradiation of* B. coli *through the Cobalt Chloride Screen.*

Exposure in minutes.	Numbers per c.c. surviving.
0	810,000
1	213,900
$1\frac{1}{2}$	92,660
2	6946
$2\frac{1}{2}$	56
3	<1

It can be seen from these tables that the wave-lengths longer than 2650 Å will destroy the bacteriophage, but a comparison with the tables given in Fig. 4 shows that, as would be expected, the destruction both of *B. coli* and of the bacteriophage takes considerably longer than with the whole spectrum.

Note.—Exposures are in seconds in Fig. 4 and in minutes in Tables III and IV.

We have now sufficient data for a quantitative comparison between the bactericidal effect of the whole spectrum (A, Fig. 7) and the portion of the spectrum (B, Fig. 7). By comparing the exposure-times necessary to produce definite degrees of destruction we can compare their efficiencies. As will be seen below, the ratio of the efficiencies of the whole spectrum (A) to spectrum (B) in destroying *B. coli* is about 4·3 : 1. If now the bacteriophage were much less susceptible than is *B. coli* to the longer wave-lengths (B) one would find that the ratio $\frac{(A)}{(B)}$ was increased.

The method of testing this point was as follows: Curves constructed in the same manner as those in Fig. 4 were drawn from the data given in Tables III and IV. From these curves and from those in Fig. 4 the following Table (V) of comparisons was made:

TABLE V.

	Rays used.	Number of seconds' exposure required to reduce the titre to—			
		1·0%.	0·1%.	0·01%.	0·001%.
B. coli	(*a*) Whole spectrum	25	30	34	37
	(*b*) Wave-lengths longer than 2650 Å	114	138	144	162
	Time ratios $\frac{(b)}{(a)}$	4·5	4·6	4·2	4·4
Bacteriophage	(*a*) Whole spectrum	50	70	100	125
	(*b*) Wave-lengths longer than 2650 Å	210	312	420	540
	Time ratios $\frac{(b)}{(a)}$	4·2	4·5	4·2	4·3

It is seen from this table that there is a striking agreement between the exposure ratios $\frac{(b)}{(a)}$ shown in the case of *B. coli* and the bacteriophage. In other words, the removal of the short wave-lengths resulted quantitatively in precisely the same effect in the case of the bacteriophage as with *B. coli*.

From the above experiments there can be little doubt that the relative efficiencies of the different portions of the bactericidal spectrum are the same in the case of the bacteriophage as in the case of bacteria. There are thus no grounds for supposing that the relation between the size of the particle and the wave-length plays any part in determining the relative insusceptibility of such bodies as the infective agent of the Rous fowl sarcoma.

SUMMARY.

Part I.—(1) The lethal effect of ultra-violet rays on *B. coli* has been investigated quantitatively, and it has been shown that the form of the survival curve under irradiation is different from that described for antiseptics and heat.

(2) Using the lethal effect upon *B. coli* as a standard, a comparison has been made between the effect of irradiation upon the bacteriophage and upon trypsin, diastase, lysozyme, complement and hæmolytic amboceptor.

It has been found that the susceptibility of these ferments and antibodies to ultra-violet rays is considerably less than that of bacteria. The susceptibility of the bacteriophage is of the same order as that of bacteria.

Part II.—(1) The relative lethal effects of three regions of the mercury vapour spectrum on *B. coli* and on the bacteriophage have been compared.

(2) The region of wave-lengths longer than 3000 Å, known to be non-bactericidal, has no lethal action on the bacteriophage.

(3) In the case of *B. coli* it was found that the relative lethal efficiencies of (*a*) the whole region 3000–2000 Å to (*b*) the region 3000–2650 Å were as about 4·4 to 1. The same ratio between (*a*) and (*b*) was found in the case of the bacteriophage.

CONCLUSIONS.

The results of this investigation indicate that ferments and antibodies are much less susceptible to the destructive effects of ultra-violet rays than are bacteria.

The susceptibility of the bacteriophage is of the same order as that of bacteria.

The relative lethal efficiencies of different regions of the spectrum are the same for the bacteriophage as for *B. coli*.

The work of Rivers and Gates (1928) indicates that the susceptibility of vaccinia virus to irradiation is of the same order as that of bacteria. The present work, therefore, strongly supports the view that the bacteriophage is of the nature of a virus and has little in common with ferments.

REFERENCES.

APPELMANS, R.—(1922) *C. R. Soc. Biol.*, Paris, **86**, 508.
BAKER, S. L., AND PEACOCK, P. R.—(1926) *Brit. J. Exp. Path.*, **7**, 310.

FISHER, R., AND MCKINLEY, E. B.—(1927) *J. Infect. Dis.*, **40**, 399.
GERETSEN, F. C., GRYNS, A., SACH, J., AND SÖHNGEN, N. L.—(1924) *Zbl. Bakt.*, Abt. II, Orig., **60**, 311.
GILDENMEISTER, E.—(1922) *Ibid.*, Abt. I, Orig., **89**, 181.
MCKINLEY, E. B., FISHER, R., AND HOLDEN, M.—(1926) *Proc. Soc. Exp. Biol.*, **28**, 408.
RIVERS, T. M., AND GATES, F. L.—(1928) *J. Exp. Med.*, **47**, 45.
YULE, G. U.—(1910) *J. Roy. Statist. Soc.*, **73**, 26.
ZOELLER, C.—(1923) *C. R. Soc. Biol.*, Paris, **89**, 860.

A SIMPLE MICROCATAPHORESIS CELL.

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DURING the last few years considerable attention has been paid, especially in the United States, to the nature of the electric charge carried by bacteria, and its significance in certain problems dealing with immunity.

In that country the majority of workers have used either the cell devised by Northrop (1922), or some modification of it. The essential feature of this cell is the use of non-polarizable electrodes which consist of pure zinc rods immersed in a saturated solution of zinc sulphate. This, by means of three-way taps, is made directly continuous with the suspension of bacteria in the cell proper.

Using this cell and its modifications Northrop and De Kruif (1922), Shibley (1924), Falk, Gussin and Jacobson (1925) have been able to determine the migration rates of bacteria in an electric field under varying conditions.

By studying the migration-rates of pneumococci, Falk and his co-workers have come to the conclusion that they are able to differentiate the four types of this organism.

There is no doubt that the migration-rate of bacteria, in an electric field, is directly dependent upon the charge carried by the organism, but the validity of the direct correlation of the potential differences between the organism and the surrounding fluid with its migration-rate, by means of the Helmholtz-Lamb equation, appears to be open to doubt.

As the result of the theoretical considerations of Winslow, Falk and Caulfield (1923) we have adopted the procedure of Falk and his co-workers (1925) of expressing the results obtained merely in terms of migration-rate without any reference to correlated potential difference.

In the earlier work on bacterial migration most observers used some form of apparatus with platinum electrodes. Russ (1909) employed both the micro- and macroscopic methods and found that most bacteria migrated at a definite rate towards the anode.

Arkwright (1914) in studying acid agglutination of *B. typhosus* noted that the charge carried by these organisms was inversely related to the degree of agglutination obtained. He used a microscope cell with platinum electrodes.

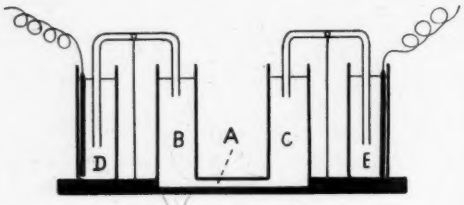


FIG. 1

In an attempt to repeat the results obtained by Northrop, Shibley and other workers, we first used a simple microscope cell with platinum electrodes, but failed to get consistent results. In our opinion this was due to two factors; first, it was noted that migration-rates showed a tendency to increase during a series of observations. This effect was apparently due to the heating of the contents of the cells by the passage of the current. The second irregularity in the results was due to polarization taking place.

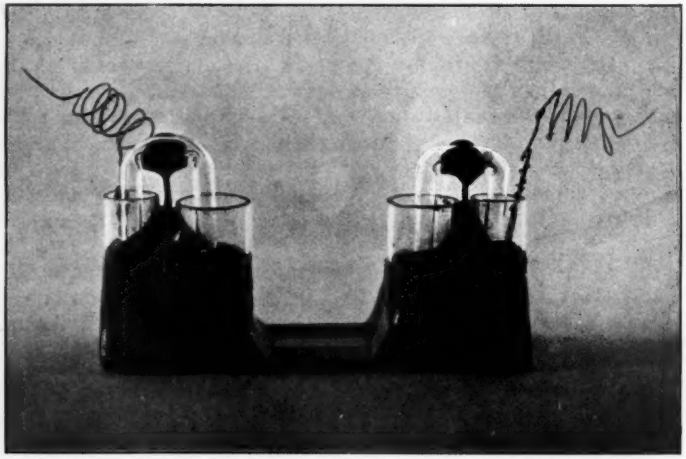


FIG. 1A.

It was therefore considered that a cell which could be easily made with non-polarizable electrodes and in which heating effects were reduced to a minimum was essential. The cell, as adopted finally, is shown both in Figs. 1 and 1A.

The Construction of the Cell.

It consists of a central cell (A) whose ends are continuous with two vertical glass tubes (B and C). Two other glass tubes (D and E), closed at the lower end, are mounted separately on the slide and contain a saturated solution of

zinc sulphate in which pure zinc electrodes are placed. As we have found great difficulty in obtaining pure zinc wire, the electrodes were made by drawing up a column of molten, pure and arsenic-free granulated zinc into a capillary pipette made of resistance glass, which was then broken off. Electrical connection between the zinc sulphate and the suspension under investigation is maintained by means of two removable inverted U-tubes containing 1% sodium chloride in 2% agar.

The base consists of a microscope slide 3 in. × 1 in.; the thickness of this is of importance and should not exceed 0·75 mm., because if it is greater than this it is not possible to use the cell for dark ground illumination.

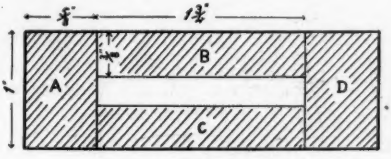


FIG. 2

The cell is made in the following way: Upon the slide four pieces of thin glass of equal thickness, approximately 0·75 mm., are cemented with bitumen (A, B, C, D, Fig. 2). Above these again six more pieces of similar thickness are cemented (E, F, G, H, I, J, Fig. 3) and finally a cover glass (K, Fig. 4) of the same thickness is cemented by means of Canada balsam.

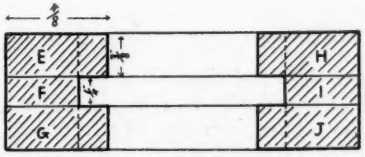


FIG. 3

The cell now consists of a shallow trough open at both ends.

Before the cover-glass is placed in position, a fine line is drawn on its under surface and another line at right angles to this on the surface of the bottom of the cell. The cover-glass and slide are coated with a thin film of paraffin wax, through this a fine line is cut by means of a razor-blade and hydrofluric acid is then applied to etch it. By means of these lines the depth of the cell can be determined.

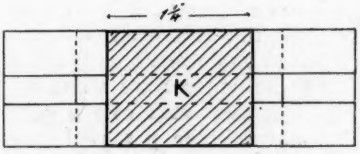


FIG. 4

The glass tubes, with an internal bore of approximately 8 mm., are now mounted, the inner pair over the open ends of the cell. Sealing-wax is suitable for this purpose. A small metal support is mounted between the inner and outer tubes; this enables the position of the U-tubes containing agar to be constant. The U-tubes have an internal bore of 3 mm., and it is advisable for the tubes which dip into the bacterial suspension to be as short as possible in order to avoid currents due to capillary attraction between the U-tube and the wall of the larger tube.

Method of Use.

The cell, which has been thoroughly washed out with freshly boiled distilled water, is filled with the bacterial suspension under investigation. It

is naturally important to be sure that there are no air-bubbles in the cell and to obviate these the open end of a rubber teat may be applied to the end of tube B or C, Fig. 1, and gentle alternate pressure and suction exerted.

The U-tubes are completely filled with the melted agar and allowed to cool to room temperature before use. The two outer tubes are filled with a saturated solution of zinc sulphate and a microammeter is joined in series in the negative lead between one of the zinc electrodes and the accumulator supplying the current. A reversing switch is incorporated in the circuit.

We have used throughout our experiments dark ground illumination, with a $\frac{2}{3}$-in. objective and × 25 ocular, a central stop being used with the ordinary Abbé condenser. Small squares calibrated by means of a stage micrometer are ruled on a coverslip which is inserted in the ocular.

Some observers take readings of the migration-rate at a number of different levels in the cell, but we have adopted the procedure of Falk (1925), which is based on the work of Smoluchowski (1921) and Mooney (1924). These observers showed that two stationary layers exist in the cell and in these the bacteria are unaffected by endosmotic streaming. These layers are located at a height of $\frac{\frac{1}{2}D}{\sqrt{3}}$ above or below the middle of the cell, where D is the depth of the cell.

The depth can be most conveniently found, as shown by Falk, Tonney, White and Jensen (1927), by setting the calibration of the fine adjustment of the microscope to zero, and then focusing the line on the under surface of the cover-glass with the coarse adjustment. The fine adjustment is then used to focus down to the other line and in this way the depth D is expressed in terms of the graduations on the fine adjustment.

The cell being filled and placed in position, the microscope is racked down to focus the stationary layer. Six observations are made, three with the switch in one direction and three in the reverse, and the times taken to cross one of the squares are noted by means of a stop watch. The mean of these results is converted into μ per second for an impressed voltage of 1 volt per centimetre. The potential gradient or impressed voltage per centimetre in the cell is found by dividing the voltage-drop by the length of the cell in centimetres between the ends of the U-tubes which are in contact with the bacterial suspension.

The instrument which we have used and found to be very satisfactory is the Dix-onemeter supplied by Messrs. Leslie Dixon & Co., 218, Upper Thames Street, E.C. 4. The price of this instrument, including the necessary multipliers and shunts, is approximately £3 10*s*. 0*d*.

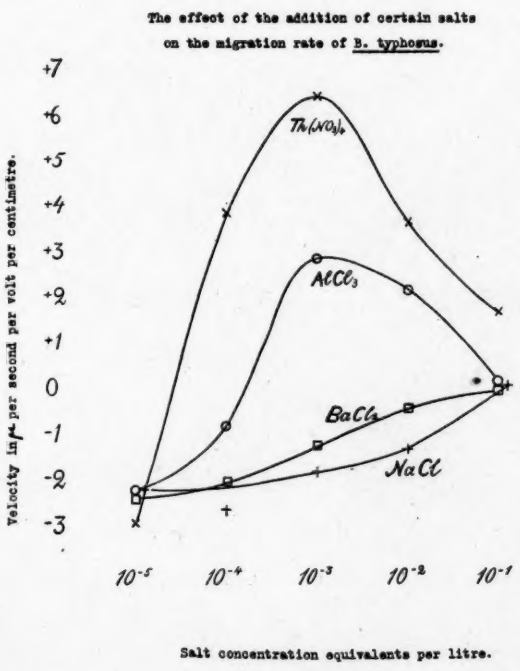
When the bacterial suspension is made in distilled water, the voltage-drop in the U-tube may be disregarded, as the resistance of the contents of the cell is of the order of $1\frac{1}{2}$ megohms. Where, however, the bacteria are suspended in solutions of salts in concentrations greater than $\frac{N}{10^4}$ the voltage drop in the U-tubes must be taken into consideration. This may be arrived at in the following manner:

The resistance of the contents of the U-tubes, apart from the cell, is obtained by measuring the amount of current which passes through the

tubes when they are joined in series with a milliammeter; a pressure of 10 volts is suitable in this case. In the cell which we have been using, the resistance of the two U-tubes was 13,500 ohms. The total current passing through the whole cell with the U-tubes in position is next determined. This naturally varies with the degree of concentration of the salt in the cell proper.

The voltage-drop in the U-tubes equals the resistance of the U-tubes multiplied by the current in ampères which passes through the whole circuit. The following table shows the voltage-drop and current passing in an actual experiment. 60 volts were used with a cell 7·6 cm. in length.

Concentration of $Th(NO_3)_4$ in equivalents per litre.	Current in circuit.	Voltage-drop in U-tubes.	Voltage-drop in cell proper.	Potential gradient in cell proper.
N. 10^{-5}	40 micro-amps.	0·54 volts	59·46 volts	7·8 volts per cm.
N. 10^{-4}	50 ,,	0·67 ,,	59·23 ,,	7·8 ,,
N. 10^{-3}	160 ,,	2·15 ,,	57·85 ,,	7·6 ,,
N. 10^{-2}	830 ,,	11·2 ,,	48·8 ,,	6·4 ,,
N. 10^{-1}	2·75 milliamps.	37·25 ,,	22·75 ,,	3 ,,
Control of distilled water	40 micro-amps.	0·54 ,,	59·46 ,,	7·8 volts.



The result of such an experiment would be expressed in μ per second per volt per centimetre, which is arrived at by dividing the breadth of the square in the ocular in μ by the time in seconds taken to traverse this square and by

the potential gradient. In the above instance of the distilled water control, where the breadth is 220 μ, the time 10 seconds and the potential gradient is 7·8 volts per cm., the migration rate is $\frac{220}{10 \times 7{\cdot}8} = 2{\cdot}82$ μ per second per volt per centimetre.

In order to test whether the results with this simple cell would correspond to those which Northrop obtained with his standard cell, a series of experiments similar to his was performed. In the first place the migration rate of a 24-hours' culture of *B. typhosus*, washed three times in distilled water in the centrifuge, was studied.

These observations were repeated on twelve successive days, and the mean of these results was found to approximate closely the figure obtained by Northrop, namely, 2·65 μ per second per volt per centimetre towards the anode, our figure being 2·99.

We also studied the effect on migration rate of adding varying concentrations of salts to a washed culture of *B. typhosus*; the results are shown in the chart herewith, whose curves closely resemble those obtained by Northrop.

SUMMARY.

A simple apparatus for the study of the migration-rate of bacteria is described for use with dark ground illumination. There is no difficulty in studying the behaviour of leptospira in this cell and further work is in progress on this subject.

We wish to express our thanks to Mr. B. Jobling for the drawings of the cell.

REFERENCES.

Arkwright, J. A.—(1914) *J. Hyg.*, **14**, 261.
Falk, I. S., Gussin, H. A., and Jacobson, M. A.—(1925) *J. Infect. Dis.*, **37**, 481, 499, 507.
Falk, I. S., Tonney, F. O., White, J. L., and Jensen, L. B.—(1927) *Amer. J. Publ. Hlth.*, **17**, 714.
Mooney, M.—(1924) *Phys. Rev.*, **23**, 396.
Northrop, J. H.—(1922) *J. Gen. Physiol.*, **4**, 29.
Northrop, J. H., and De Kruif, P.—(1922) *Ibid.*, **4**, 639.
Russ, C.—(1909) *Proc. Roy. Soc.*, B., **81**, 314.
Shibley, G. S.—(1924) *J. Exp. Med.*, **40**, 453.
Smoluchowski, M. v.—(1921) Graetz's 'Handbuch der Elektrizität und des Magnetismus,' **2**, 366.
Winslow, C.-E. A., Falk, I. S., and Caulfield, M. F.—(1923) *J. Gen. Physiol.*, **6**, 177.

A SIMPLE METHOD FOR DETERMINING THE ELECTRICAL CHARGE CARRIED BY VIRUS PARTICLES.

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IT is well known that if aqueous solutions of methylene blue and eosin are dropped on filter-paper, the blue stain does not pass into the paper with the water, whereas the eosin does. The same phenomenon can be demonstrated by dipping strips of filter-paper into the stain solutions, when the eosin will travel up the strip with the water a considerable way, the methylene blue never rising more than a centimetre above the stain level. This phenomenon is due to the fact that the paper is negatively charged and the methylene blue particles positively charged, leading to a firm adsorption of the stain on the paper and thus preventing it being carried along with the water. Eosin being negatively charged is not prevented from travelling into the paper. Since the dispersion of virus particles in a virus suspension approximates to that of colloids it seemed possible that this phenomenon might be made use of in determining the charge carried by virus particles.

TECHNIQUE.

The following method has been evolved. Blotting-paper has been made use of in preference to filter-paper, because it is stiffer and holds more fluid. Each sample is tested with methylene blue and eosin before being taken into use. Strips of this paper are cut out 0·5 cm. broad × some 18·0 cm. long, and starting 0·5 cm. from one end, marked in pencil with a centimetre scale up to 10·0 cm. They are then sterilized. The virus suitably diluted with the requisite buffer is placed in a glass receptacle—watch-glass or short wide-mouthed tube—which stands on the base of a burette stand and a strip of blotting-paper, held at one end in the clamp on the stand, is carefully lowered so that the free end dips into the virus to the extent of 0·5 cm. The water is allowed to rise up to the 10 cm. mark (9·0 cm. in the vaccinia experiments), the strip withdrawn and the wetted portion cut into sample lengths, each one being dropped into a numbered sterile tube. In our earlier experiments the sampling was done at intervals of 2·5 cm., but later the wetted portion has been cut up into centimetre portions. The first centimetre above the virus need not be tested, for virus will certainly be there whatever the charge and it is unnecessary to test the last three centimetres of the wetted strip because even eosin fails to penetrate as far as this under the conditions of the experiment. The presence of virus in the centimetre lengths 2–6 is then tested for

by animal inoculation. The method employed in making these tests will, of course, depend on the virus, but where one is dealing with a virus which takes readily in the epilated skin of the test animal, one has merely to rub the sample centimetre on a previously scarified area of skin. It is important to add two or three drops of buffer to the sample portions to prevent their drying up before inoculation can be carried out. Since methylene blue and eosin only behave in the manner described when their concentration does not exceed 0·1 %, it is important not to use the virus in too great a concentration. From experience it would appear quite safe to use concentrations representing 100 to 200 times the titre. The buffers employed in the virus experiments have been :

KH_2PO_4/K_2HPO_4 M/50; pH 6·6, 7·0 and 7·6.
Borax/boric acid ; pH 8·0.

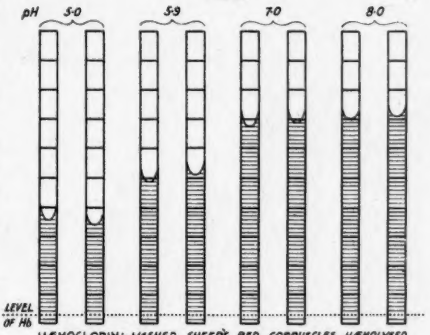
In many instances the virus suspension has been made by grinding the virus-containing tissue with sand and suspending directly in the requisite buffer. Where this has been done each separate virus has been titrated, the same buffer being used for the dilutions as was used in making the original suspensions. Since no appreciable difference has been observed between the titres given by virus suspensions made at the four different hydrogen-ion concentrations, it is safe to conclude that differences in behaviour in the charge experiments cannot be attributed to any hypothetical effect of hydrogen-ion concentration on the virulence of the virus. In other experiments the initial virus suspension was made in distilled water and the titre having been ascertained, dilutions made with the requisite buffer for each experiment. Here viability tests were made on the virus dilutions actually used in the charge experiment. On one or two occasions the virus dilutions made with the boric acid/borax buffer pH 8·0 have given poorer viability tests than those obtained with the dilutions made at the other pH with phosphate buffers. It occurred to us that possibly the protein present in the virus suspension might interfere with the experiment, but methylene blue and eosin added to virus suspensions at the various pH behaved exactly as they did in water.

RESULTS OBTAINED.

In the case of methylene blue and eosin, where the particles are strongly charged the experiment is a simple matter ; one has merely to find out whether the stain passes into the paper or is held at the bottom of the strip, an all or nothing result. But it is conceivable that in the case of a virus at a given pH one might be close to, but on the acid side of, its isoelectric point. Here the particles would be weakly positively charged and their behaviour would be something between that of eosin and methylene blue. An isolated reading in the case of a virus might not, therefore, give very much information. Here a number of readings over a range of pH would be required and the range chosen has been pH 6·6, 7·0, 7·6 and 8·0. On the alkaline side of the isoelectric point similar readings should be obtained whatever the pH., but on the acid side the virus particles should pass less and less further into the paper as the reaction became more acid until a point was reached where the particles were strongly positively charged and behaved like methylene

blue. To test this an experiment was performed with hæmoglobin the isoelectric point of which is known and the results, rendered graphically in Fig. 1, clearly support this line of reasoning.

FIG. I.

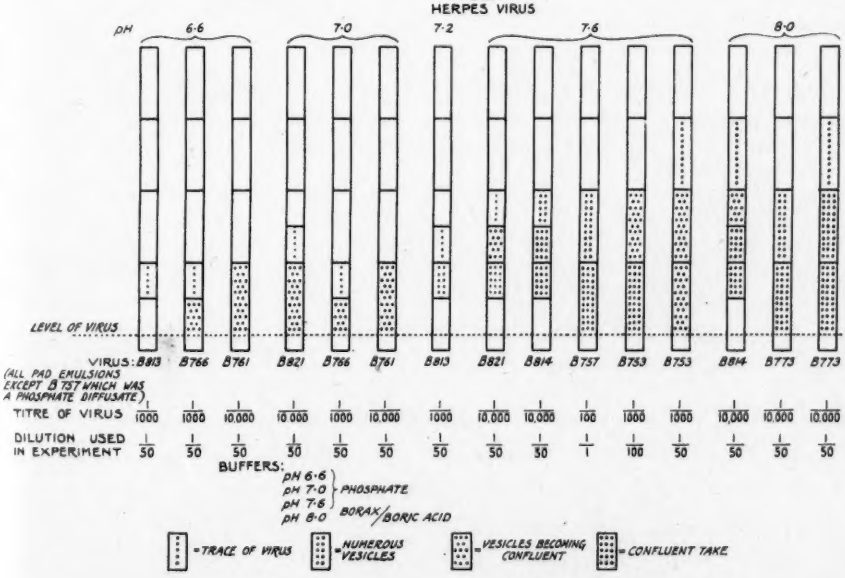


HÆMOGLOBIN: WASHED SHEEP'S RED CORPUSCLES HÆMOLYSED BY ADDITION OF 9 PARTS OF DISTILLED WATER. MADE UP TO 1 IN 1000 WITH APPROPRIATE BUFFER.
BUFFERS: pH 5·0 PHTHALATE. pH 5·9 & 7·0 PHOSPHATE. pH 8·0 BORAX/BORIC ACID.
PRESENCE OF Hb IN STRIP DEMONSTRATED BY BENZIDINE REACTION.

With viruses one cannot hope for such sharp end-points as in the case of hæmoglobin. One can merely say that in a given sample of the wetted strip of paper virus was or was not present, and if present in what quantity. This being so it would seem fair to take as the end-point the upper limit of that sample of the strip into which virus had entered in quantity. If this be allowed then the results obtained in the experiments carried out with herpes and vaccinia viruses—rendered graphically in Figs. 2 and 3—are quite clear cut and easy of interpretation.

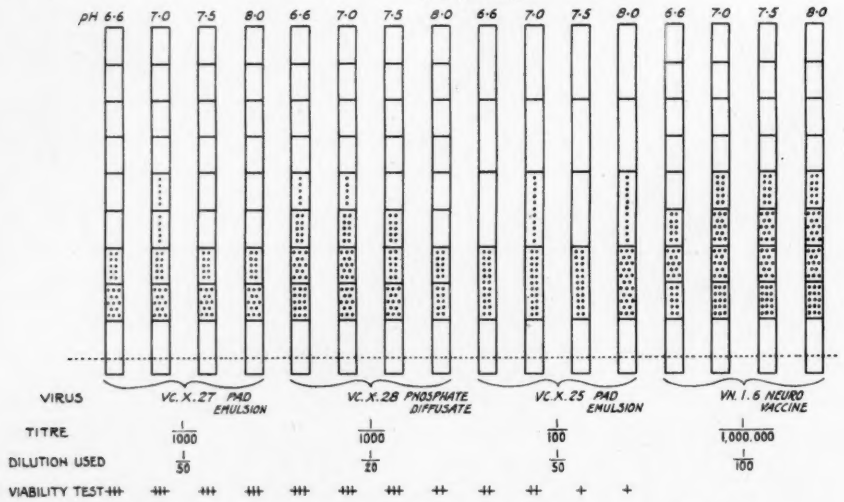
In the case of vaccinia, similar readings were obtained over the range of pH 6·6–8·0. The occasional slight discrepancies have no regular distribution. Since the virus passes well into the strip, then one may conclude that vaccinia virus is negatively charged over this range of pH, a finding in keeping with

FIG. II.
HERPES VIRUS


pH
6·6
7·0
7·2
7·6
8·0
LEVEL OF VIRUS
VIRUS: B813 B766 B761 B821 B766 B761 B813 B821 B814 B757 B753 B753 B814 B773 B773
(ALL PAD EMULSIONS EXCEPT B 757 WHICH WAS A PHOSPHATE DIFFUSATE)
TITRE OF VIRUS 1/1000 1/1000 1/10,000 1/10,000 1/1000 1/10,000 1/1000 1/10,000 1/10,000 1/100 1/1000 1/1000 1/10,000 1/10,000 1/10,000
DILUTION USED IN EXPERIMENT 1/50 1/50 1/50 1/50 1/50 1/50 1/50 1/50 1/50 1/1 1/100 1/50 1/50 1/50 1/50
BUFFERS:
pH 6·6 } PHOSPHATE
pH 7·0 }
pH 7·8
pH 8·0 BORAX/BORIC ACID
= TRACE OF VIRUS
= NUMEROUS VESICLES
= VESICLES BECOMING CONFLUENT
= CONFLUENT TAKE

the cataphoresis experiments of Douglas and Smith (1928). With herpes virus similar readings were obtained at pH 7·6 and 8·0 the virus passing well into the paper, whereas at 7·2, 7·0 and 6·6 the readings were much lower, there being an indication that at pH 6·6 the virus passed a less distance into the paper than it did at pH 7·0 and 7·2. We would interpret these findings with herpes virus as indicating that this virus is negatively charged at pH 7·6 and 8·0 and positively charged at pH 7·2, 7·0 and 6·6. The isoelectric point would thus lie between 7·6 and 7·2. Cataphoresis experiments have not been carried out with herpes virus so far as we know, but we propose doing so in the near future.

FIG. III.
VACCINIA VIRUS



The immediate importance of knowing the charge carried by virus particles is, of course, in the interpretation of filtration experiments. The filters in use are all negatively charged, and although opposite electrical charge may not be the only factor governing adsorption of particles at a surface, it is a most important one. It follows, therefore, that unless the charge carried by the virus particles under the condition of experiment is known, an important factor is left uncontrolled and a serious source of error introduced. Much filtration work has been rendered valueless owing to the questions of pH and charge being overlooked. The method which we have evolved is simple, requires no expensive or elaborate apparatus, and would appear to give reliable information. The use of this method in the separation of viruses, their purification or concentration are possibilities which suggest themselves.

REFERENCE.

DOUGLAS, S. R., AND SMITH, W.—(1928) *Brit. J. Exp. Path.*, **9**, 213.

THE EXHAUSTION OF MEDIA IN BACTERIAL CULTURE.

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It is well recognized that, if a micro-organism be cultured on nutrient agar for some days and the resultant growth washed off, no further growth will occur when the same organism is reinoculated on the slope. This inhibition by previous growth is, in some instances, manifested not only towards the same organism but also to a greater or less extent towards various other bacteria; in other cases the action appears to be strictly specific.

One of the earliest workers in this field was Chatterjee (1908) who employed this phenomenon as the basis of a test for the differentiation of members of the typhoid group.

Is the inhibitory action due to the formation of diffusible toxin?—Eijkman (1904–1906) showed that a culture of *B. coli* would inhibit not only its own growth but also that of a number of other bacteria, and he concluded that a thermolabile diffusible auto-toxin was produced during growth. He states, however, that he was unable to demonstrate any inhibition when liquid media were employed.

This work was repeated and amplified by McLeod and Govenlock (1921) who used a modification of Eijkman's technique. These observers also concluded that a thermolabile diffusible toxin had been produced during metabolism. In contradistinction to Eijkman, however, they found that the same result was obtained with broth when it was incubated in a thin layer in a wide-bottomed flask and suggest that the difference is due to better aeration.

Is the inhibitory action due to the exhaustion of accessory food factors?—It has recently been shown by Palevici (1927) that this inhibitory effect could be overcome by the addition of a few drops of lemon-juice to agar slopes on which staphylococcus had been grown for three days. No difference was noted whether the juice was incorporated in the medium after the slope had been liquefied, or was merely allowed to flow over the surface: when grape-, apple- or pear-juice was substituted for lemon, and *B. coli* or *B. paratyphosus* B. used instead of staphylococcus, the results were exactly the same. He assumed that these fruit juices provided vitamines or food accessory substances necessary for bacterial growth, in place of those exhausted by the primary inoculation.

The accessory food requirements of different organisms have been the subject of a great deal of investigation, and while Doryland (1916) was able to cultivate some bacteria on purely synthetic media, apparently containing no vitamines, other observers have found that certain micro-organisms require very definite accessory food substances.

Flack (1916), Lloyd (1916), Shearer (1917), Ayers and Rupp (1918),

Huntoon (1918) were all of opinion that vitamines play an essential part in the stimulation of growth of meningococci and streptococci.

Davis (1917) showed that there were two factors necessary for the cultivation of Pfeiffer's bacillus: one of these was present in hæmoglobin and the second in other bacteria, and in plant and animal tissues. Thjötta and Avery (1921) found that blood could supply both of these factors, but that one, which they call "V" (vitamine) was easily destroyed by heat, while the other "A" was thermostable. The thermolabile, or "V" constituent could be replaced by an extract of yeast or vegetable tissue, for they found that such an extract would reactivate blood in which the "V" factor had been destroyed by heat.

As regards the non-hæmophilic bacteria, Davis (1918) found a much heavier growth in media containing germinated grain than in normal controls, but came to the conclusion that part of the increase at least was due to the presence of a greater amount of sugars and amino acids in the sprouted grain.

Broadhurst (1921) carried out an experiment to show that old cultures could be "revivified" by the addition of vitamines to the broth in which they were subcultured, but Werkman (1927) states that the increase of growth obtained occurs only during the lag phase, that it can be directly related to the amount of nitrogen added as "vitamine concentrate" and that it ceases as soon as the nitrogen is used up. Further, he was able to demonstrate the same stimulation when the vitamines had been entirely removed by extracting the concentrate with hot alcohol for 3 weeks.

Conversely there is some evidence for assuming that certain bacteria are capable of synthesizing vitamines in a vitamine-free medium (Pacini and Russell, 1918; Werkman, 1927).

EXPERIMENTAL WORK.

As the association of vitamines on the one hand, or of a diffusible toxin on the other, does not seem to explain adequately this inhibitory action, the following experiments were performed in order to attempt to throw further light on this phenomenon.

The term "vaccinated slope" is used, as employed by French writers, to denote an agar slope on which an organism has been cultured for 72 hours and the growth washed off with distilled water; *B. typhosus* Rawlings is the organism employed, and 0·5 c.c. the amount of solution used unless stated otherwise.

In the first place, a series of *B. typhosus*-vaccinated slopes was prepared and the following bacteria inoculated on them.

Second organism inoculated.	Growth.
Staphylococcus aureus	+
B. prodigiosus	+
B. paratyphosus B	+
B. ærtrycke Mutton	+
B. coli Escherich	+
B. typhosus Rawlings (control)	0

+ = growth; 0 = no growth.

Growth was most marked with staphylococcus and *B. prodigiosus*, while in the controls there was no sign at all of growth. This is in exact accordance with the findings of Chatterjee.

Palevici's experiment was repeated and confirmed in the following manner: 0·5 c.c. of fresh orange-juice was added to each of another series of *B. typhosus*-vaccinated slopes, half of the number having been liquefied and the juice added before re-setting. All were reinoculated with *B. typhosus* and incubated for 48 hours. Good growth occurred in every case, while controls, which had been liquefied and re-sown without the addition of orange-juice, showed no growth.

Is the reactivating power of orange-juice due to its vitamine content?— The destruction of vitamines by heat depends on so many factors, as the source from which they are obtained, the reaction of the medium in which they are present, and the presence or absence of a free supply of oxygen during the heating process, that it appears impossible to state that all are destroyed at any specific temperature, and especially when the containing medium, as orange-juice, is acid (Funk, 1922; Plimmer and Plimmer, 1922; Ellis and MacLeod, 1923). Plimmer and Plimmer, however, note that vitamines are very readily carried down by any precipitate, and Lloyd (1916) found that they are almost entirely adsorbed by filtration through paper.

Thjötta and Avery (1921) found that the growth-stimulating factors for *B. influenzæ* present in tomato-juice and in yeast extract are totally destroyed by autoclaving at 120° C. for 30 minutes.

The following experiment was carried out in an attempt to destroy the factor in orange-juice responsible for the regeneration of growth.

About 20 c.c. of fresh juice was filtered three times through paper, and one half of the filtrate brought to pH 8·0 with N/1 NaOH. Some unfiltered juice was also neutralized and the four types, viz., acid unfiltered, neutral unfiltered, acid filtered and neutral filtered, were autoclaved at 125° C. for 3 hours. Their growth-regenerating powers were tested as before and compared with the same types which had not been autoclaved.

		Growth on second inoculation.
Vaccinated slope	+ acid unfiltered juice	+
,,	+ neutral ,, ,,	+
,,	+ acid filtered ,,	+
,,	+ neutral filtered ,,	+
,,	+ acid unfiltered autoclaved	+
,,	+ neutral ,, ,,	+
,,	+ acid filtered ,,	+
,,	+ neutral filtered ,,	+
,,	(control)	0

+ = growth; 0 = no growth.

i. e., a temperature of 125° C. for 3 hours, combined with previous filtration and neutralization, does not destroy the growth-regenerating power of orange-juice.

To test whether this factor could be removed by precipitation, 5 c.c. of

fresh juice was added to 15 c.c. of absolute alcohol and the resultant precipitate filtered off, washed with alcohol, and redissolved in 5 c.c. of distilled water. The filtrate was evaporated down to remove the alcohol, and made up to 5 c.c. with distilled water.

Another 5 c.c. of juice was shaken with ether and, after standing, the upper fraction was pipetted off, evaporated to dryness, and the residue dissolved in 5 c.c. of distilled water.

0·5 c.c. of each was added to *B. typhosus*-vaccinated slopes as before, with the following results:

		Growth on second inoculation.
Vaccinated slope	+ re-dissolved alcoholic precipitate .	+
,,	+ alcohol-free filtrate	+
,,	+ re-dissolved ether-soluble fraction .	0
,,	+ ether-insoluble fraction . . .	+
,,	(control)	0

+ = growth; 0 = no growth.

From the above it would appear that the active principle, although soluble in water and alcohol and insoluble in ether, is not of the nature of a vitamine.

Is any other substance present in orange-juice the activating principle?—The analysis of Californian oranges, made by Willmot (1928) shows a very high percentage of reducing sugar in the total solids (10·8% after hydrolysis to 12·8% total solids).

In order to investigate the importance of this in the problem under discussion, the action of Fehling's solution upon the aqueous, alcoholic and ethereal extracts above mentioned was tested, and it was found that the aqueous and alcoholic extracts, both of which were active, gave a strongly positive reaction, while with the inert ethereal extract the reaction with Fehling's was negative.

In an attempt to remove the sugar, *B. coli* was grown in neutralized orange-juice, but reducing sugar could still be demonstrated after one week's incubation at 37° C.

The growth-regenerating power of glucose was therefore tested, decreasing amounts being added to vaccinated slopes, with the results shown below.

The percentage of glucose is the final concentration in the medium.

		Growth on second inoculation.
Vaccinated slope	+ 1·0% glucose	+
,,	+ 0·5% ,, ...	+
,,	+ 0·1% ,, ...	+
,,	+ 0·05% ,, ...	±
,,	+ 0·01% ,, ...	trace
,,	(control)	0

+ = growth; ± = slight growth; 0 = no growth.

i. e., the addition of glucose to a *B. typhosus*-vaccinated slope will allow growth to recur.

Galactose, mannose, and lævulose were tested in the same manner and gave exactly the same results, while maltose, which the Rawlings strain of *B. typhosus* does not ferment, was inactive in this respect.

A further series of sugars was prepared and tested with various bacteria of the coli-typhoid group, specifically vaccinated slopes being used and the sugar added in amounts to give a final concentration of 1 % in each case.

		Second inoculation, *B. fæcalis alcaligenes.* Growth.
Vaccinated slope	+ glucose	0
,,	+ maltose	0
,,	+ saccharose	0
,,	+ lactose	0

		Second inoculation, *B. typhosus* Rawlings. Growth.
Vaccinated slope	+ glucose	+
,,	+ maltose	0
,,	+ saccharose	0
,,	+ lactose	0

		Second inoculation, *B. coli.* Growth.
Vaccinated slope	+ glucose	+
,,	+ maltose	+
,,	+ saccharose	0
,,	+ lactose	+

		Second inoculation, *B. cloacæ.* Growth.
Vaccinated slope	+ glucose	+
,,	+ maltose	+
,,	+ saccharose	+
,,	+ lactose	+

The technique adopted was as follows: 20 % solutions of the sugars were prepared and sterilized by filtration through a Pasteur-Chamberland L5 candle because it was found that saccharose, which is composed of glucose and lævulose, autoclaved at 120° C. for 20 minutes, would resuscitate a *B. coli*-vaccinated slope while it was inactive in this respect when sterilized by filtration. The slopes were liquefied and allowed to cool to 50° C. before the sugar was added.

The effect of salts of organic acids was next tested in this respect, and with them the same type of result was obtained, viz., growth recurred when the organism was able to utilize the salt, as shown in the tables.

			Second inoculation, *B. fæcalis alcaligenes* Growth.
Vaccinated slope	+	sodium citrate	0
,,	+	,, dextrotartrate . .	0

			Second inoculation, *B. paratyphosus* B. Growth.
Vaccinated slope	+	sodium citrate	+
,,	+	,, dextrotartrate . .	0

			Second inoculation, *B. coli*. Growth.
Vaccinated slope	+	sodium citrate	0
,,	+	,, dextrotartrate . .	+

The addition of 0·5 %, final concentration, of peptone to vaccinated slopes allowed growth to recur in every case, but ammonium potassium phosphate had no such effect, although tried in a number of different dilutions.

The Effect of Adding Utilizable Carbohydrate before Primary Inoculation.

To test whether the addition of glucose to a medium before the original inoculation would allow secondary growth of the same organism to occur, glucose was added to nutrient agar slopes in amounts to give final concentrations of 2% and 1% respectively. These slopes, together with normal agar controls, were sown with *B. coli* Escherich and incubated for 48 hours at 37° C.

The growth was washed off, the reaction readjusted to pH 7·4. All were reinoculated with *B. coli* and incubated for a further 72 hours.

Growth had recurred in every case except the normal agar controls: the slope which originally contained 1% of glucose was alkaline to phenol red while the other was acid.

As before, the slopes were washed, their reactions adjusted, and they were re-sown with *B. coli*.

The table shows the results obtained, each incubation being for 72 hours:

	First inoculation.		Second inoculation.		Third inoculation.		Fourth inoculation.	
	Growth.	Reaction.	Growth.	Reaction.	Growth.	Reaction.	Growth.	Reaction.
Control	+	Alk.	0	...	...	...	...	...
1% glucose	+	Acid	+	Alk.	0	...	...	...
2% ,,	+	,,	+	Acid	+	Alk.	0	...

+ = growth; 0 = no growth; alk. = alkaline.

Growth can, therefore, recur on second inoculation if additional carbohydrate be provided before the first sowing; but, since the presence of glucose causes a rapid rise in the hydrogen ion concentration of the medium during growth, it may be argued that growth, and metabolic products in the medium, are interfered with, so that the "inhibitory substances" are not formed as they would be under normal conditions.

Smaller percentages of glucose were therefore employed and it was found that 0·25 %, added to the medium before original inoculation, was the highest dilution which would allow growth to recur. In this case the reaction of the medium remained alkaline during 72 hours incubation, but as Ayers and Rupp (1920) have shown that acid and alkaline fermentations can occur simultaneously in a medium, that does not mean that the glucose is not utilized.

To test this contention further, sodium citrate was added to agar slopes in 2% and 1 % dilutions, and the slopes were inoculated with *B. lactis ærogenes* and incubated at 37° C. for 72 hours. When the growth was washed off and the slopes resown with the same organism, good growth was obtained although the reaction of the medium had remained alkaline throughout the experiment.

The effect produced by the addition of glucose is not due to any alteration in hydrogen ion concentration which occurs, since the same effect is obtained when sodium citrate is the source of carbon.

It would appear, therefore, that the presence, in the reactivating substance, of carbon in a form available to the organism plays an important part in the action.

The Inhibitory Effect of Growth of Bacteria upon the Subsequent Growth of Other Bacteria.

If the recurrence of growth depends on available carbon, it seems possible that some relation may exist between the number of "sugars" fermented by an organism and the number of other bacteria whose growth it will inhibit.

Effect of Similar Organisms with Different "Sugar" Reactions.—Although *B. typhosus* Rawlings is a strain frequently used, mainly on account of its antigenic properties, the strain with which I worked is by no means classical in its behaviour towards the fermentable carbohydrates. Even with prolonged incubation it persistently fails to ferment maltose.

It therefore appeared that additional light might be thrown on this matter by comparing the action of this strain with other strains which ferment maltose in the ordinary way.

For several of these I am indebted to Dr. J. T. Duncan.

Vaccinated slopes of a number of different strains were prepared and the various strains cross-inoculated.

From the table it will be seen that strain Rawlings, which does not utilize maltose, has no inhibitory action on the strains which do ferment that sugar.

Second inoculation.	First inoculation.			
	B. typhosus Rawlings.	*B. typhosus* Hy.	*B. typhosus* Hd.	*B. typhosus* W.
B. typhosus Rawlings	0	0	0	0
,, Hy.	±	0	0	0
,, Hd.	±	0	0	0
,, W.	±	0	0	0

± = slight growth; 0 = no growth.

Effect of Different Organisms with the Same "Sugar" Reaction. The inhibition shown by members of the Salmonella group having the same sugar reactions was next tested in the same manner.

Vertically these tables show the inhibitory power of the bacteria originally inoculated. Horizontally they show their capacity to grow on slopes "vaccinated" by other organisms.

Second inoculation.	First inoculation. *B. paratyphosus* A.	*B. paratyphosus* B.	*B. paratyphosus* C.	*B ærtrycke* Mutton.
B. paratyphosus A	0	0	0	0
„ B	±	0	0	0
„ C	±	0	0	0
B. ærtrycke Mutton	±	±	±	0

± = slight growth; 0 = no growth.

The above results are somewhat conflicting as all these bacteria ferment glucose, maltose and mannitol, and it is not clear why the inhibitory action of *B. paratyphosus* A is distinctly weaker than that of *B. paratyphosus* B, while *B. ærtrycke* Mutton shows the strongest inhibition. If, however, the action of these organisms upon the organic salts is taken into account (Brown, Duncan and Henry, 1924) the explanation appears obvious.

The following table shows the utilization of certain organic salts as given by these workers.

	Sodium citrate.	Sodium dextrotartrate.	Sodium lævotartrate.
B. paratyphosus A	0	0	0
„ B	+	0	+
„ C	+	+	0
B. ærtrycke Mutton	+	+	+

+ = utilization of salt; 0 = no utilization of salt.

Here *B. paratyphosus* A is inert to citrate, dextrotartrate and lævotartrate of soda, whereas *B. ærtrycke* Mutton ferments all three salts; *B. paratyphosus* B and C are intermediate in their action.

This finding is in exact accordance with the inhibitory action here shown.

To investigate further this apparent relationship between inhibitory power and the number of carbohydrates fermented by an organism, a series of bacteria was taken whose members ferment an increasing number of sugars, and their inhibitory effect on each other tested as before.

As the results in this experiment were anomalous and not clearly defined, a similar experiment was performed in which the first incubation was carried on for 7 days, the growth being then washed off and the slopes cross-inoculated as before.

The results are given in the following table:

Second inoculation.	First inoculation.							
	B. fæcalis alcaligenes.	*B. dysenteriæ* Shiga.	*B. Morgan* No. 1.	*B. typhosus* Rawlings.	*B. typhosus* Hy.	*B. paratyphosus* B.	*B. coli* Escherich.	*B. lactis ærogenes.*
B. fæcalis alcaligenes	0	+	+	+	+	+	+	+
B. dysenteriæ Shiga	+	0	+	0	0	0	0	0
B. Morgan No. 1	+	+	0	+	+	0	0	0
B. typhosus Rawlings	+	+	0	0	0	0	0	0
,, Hy.	+	+	0	0	0	0	0	0
B. paratyphosus B	+	+	+	+	+	0	0	0
B. coli Escherich	+	+	+	+	+	0	0	0
B. lactis ærogenes	+	+	+	+	+	+	+	0

+ = growth; 0 = no growth.

As is shown in the table, there is a marked increase in the inhibitory effect when the first incubation is allowed to continue for seven days.

It is worthy of note that neither *B. fæcalis alcaligenes* nor *B. lactis ærogenes* is inhibited by any of the bacteria tested. Winslow, Kligler and Rothberg (1919), from an extensive study of the carbohydrate fermentation reaction of the coli-typhoid group, consider *B. lactis ærogenes* to be the most primitive member, and hence able to utilize substances which are not available to the others. With regard to *B. fæcalis alcaligenes*, it seems more probable that it is able to grow after the others because its carbohydrate requirements are entirely different from theirs, than that it is immune to a "toxin" produced by their metabolism.

The most marked inhibitory effects are shown by *B. coli*, *B. paratyphosus* B and *B. lactis ærogenes*, and by testing the effect of the whole series on the organic salts it was found that these are the only members which ferment any of the salts employed.

Since it was noted in an earlier experiment that the inhibitory effects of members of the Salmonella group appeared to be related to their power to ferment these salts, the inhibitory action towards the above series shown by *B. paratyphosus* A, *B. paratyphosus* C and by *B. ærtrycke* Mutton, after growth for seven days, was next tested.

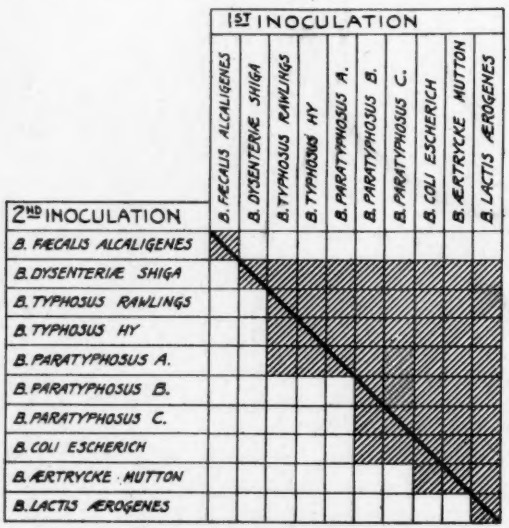
As shown in the table (p. 80), the inhibitory action increases from *B. paratyphosus* A to *B. ærtrycke* Mutton as before.

The foregoing experiments on inhibition were repeated and amplified to give a complete table. The findings are shown diagrammatically (p. 80); the shaded squares represent inhibition of the second organism by the one originally inoculated, and the unshaded squares growth of the second bacterium. As with the tables, the diagram shows, vertically, the inhibitory effect of an organism when it is the first inoculated, and, horizontally, its power of growth when inoculated after the others.

Second inoculation.	First inoculation. *B. paratyphosus* A.	*B. paratyphosus* C.	*B. ærtrycke* Mutton.
B. fæcalis alcaligenes	+	+	+
B. dysenteriæ Shiga	0	0	0
B. Morgan No. 1	+	+	0
B. typhosus Rawlings	0	0	0
„ Hy.	0	0	0
B. paratyphosus B	+	0	0
B. coli Escherich	+	0	0
B. lactis ærogenes	+	+	+

+ = growth; 0 = no growth.

If the fermentative activities of these bacteria towards "sugars" and organic salts be considered in relation to the diagram, it seems apparent that a definite relationship exists between the power of an organism to utilize the carbon present in a medium and its capability of growth when inoculated on a slope on which another organism has previously been cultivated.



The importance of taking into consideration the actions of the bacteria on the organic salts in addition to the "sugars" is well shown by the differences in inhibitory effect, towards the other bacteria of the series as well as to each other, exhibited by *B. paratyphosus* A, *B. paratyphosus* B and *B. ærtrycke* Mutton.

This factor may also explain why *B. coli* Escherich is more easily inhibited than *B. ærtrycke* Mutton, because, although *B. coli* ferments lactose in addition to the three carbohydrates, glucose, mannitol and maltose, fermented by *B. ærtrycke* Mutton, the latter is much the more active towards the organic salts.

Effect of Filtrate of Broth Cultures on Fresh Media.

To determine whether the inhibition is due to toxin formation, the effect of adding to fresh peptone water increasing amounts of a 6-day broth culture of *B. typhosus* Rawlings, filtered through an L5 Pasteur-Chamberland candle, was tested. The culture was incubated in small quantities in wide-bottomed flasks as advised by MacLeod and Govenlock.

As a preliminary, to obviate a possible source of error due to the mechanical dilution of the medium with the filtrate, the amount of growth obtained at 24-hours, with equal quantities of inoculum of *B. typhosus*, in different percentages of peptone was standardized by the opacity method (Brown, 1919).

As this showed a marked and fairly regular increase in numbers with an increasing percentage of peptone, fresh peptone solution was added to the filtrate so that the final concentration in each case was 1·5%. The media were inoculated with 0·035 c.c. of a 24-hour broth culture of *B. typhosus* incubated at 37 C. for 24 hours, and growth standardized as above.

Percentage of peptone.	Percentage of contained filtrate.	Weight of dried bacterial substance per c c.
1·5 (control)	...	0·58 mgm.
,,	10 (1/10)	,,
,,	20 (1/5)	,,
,,	25 (1/4)	,,
,,	50 (1/2)	,,

Control of filtrate only remained clear.

Using this method of standardization, the opacities of the cultures containing filtrate and peptone were indistinguishable from that of the control. Therefore the presence of the filtrate does not inhibit the growth of the organism in fresh peptone as it should do if toxic products were present as the result of metabolism.

DISCUSSION.

The two theories which have been brought forward to explain the inability of a "vaccinated" slope to support further growth of the organism are (1) that a specific auto-toxin is produced during metabolism, and (2) that there is an exhaustion of the vitamines originally present in the medium.

As regards the former, it has been impossible to demonstrate any inhibition of subsequent growth when the filtrate of a 6-day broth culture of *B. typhosus* was added to fresh peptone. In experiments on inhibitory effect shown by members of a series towards each other, it was always found that those whose power of utilizing carbon compounds was limited were much more easily inhibited than those which could assimilate carbon from a greater number of sources.

That the inhibition is not due to an exhaustion of vitamines has been proved definitely since, whatever be the position as regards the requirements, or production, of vitamines by the coli-typhoid group, the effect of fruit juice on vaccinated slopes is not due to its vitamine content but to the presence in it of fermentable carbon compounds. The same results are obtained when peptone, or a sugar or organic salt which the organism can ferment, is substituted for the fruit juice.

In this connection it is of interest that Perrotti (quoted by Doryland, 1916) states, with regard to the utilization of cyanide nitrogen by certain bacteria, that "the assimilation of either ammonia, nitrate or cyanide nitrogen is dependent, always, upon the presence of suitable energy materials."

Similarly, Waksman (1920) holds that a definite amount of ammonia may be formed from complex nitrogenous compounds whether available carbohydrates be present or not, but that in the presence of the sugar the ammonia may be utilized, while in carbohydrate-free media proteolytic organisms utilize the protein as a source of carbon as well as of nitrogen.

The addition of available carbohydrate to a medium before primary inoculation would, therefore, exert a "sparing" action on the protein, thereby leaving a sufficiency of energy material to allow growth to occur on second inoculation. Addition after primary growth has taken place would enable the organism to assimilate nitrogen from sources which, otherwise, would be unavailable to it, and hence growth can recur.

In the inhibitory effects considered here, the factor which determines the appearance of inhibition appears to be the relative powers of the organisms to assimilate carbon from different sources: *e.g.*, *B. lactis ærogenes*, whose fermentative activities are very great, removes from the medium such energy materials as are available to the other members of the group, while the same activity enables it to grow on slopes which have been "vaccinated" by these bacteria.

The only exception to this rule occurs with *B. fæcalis alcaligenes* which is not inhibited even by *B. lactis ærogenes* although it is completely inactive towards the carbohydrates, but it seems probable that its behavour is due to the manner in which its entire metabolism differs from the others.

SUMMARY.

It has been shown that the power of fruit juice to allow of regrowth of an organism on an agar slope on which that organism has already been grown for some days, is dependent, not on its vitamine content, but on the presence of carbon compounds which the organism can assimilate.

With regard to the coli-typhoid group, certain evidence has been brought forward to show that the power of a bacterium to inhibit the growth of others is related to its fermentative activities of "sugars" and organic salts.

It is suggested that this inhibition is caused by a deficiency of available carbon due to the original growth, and that this explains why bacteria with greater powers of fermentation inhibit those which are less active in this respect.

I should like to record my thanks to Major Brown, I.M.S., at whose suggestion this investigation was originally undertaken, for much valuable advice and criticism.

REFERENCES.

Ayers, S. H., and Rupp, P.—(1918) *J. Infect. Dis.*, **23**, 188.—(1920) *J. Bact.*, **5**, 8.
Broadhurst, J.—(1921) *Abst Bact.*, **5**, 3.
Brown, H. C.—(1919) *Ind. J. Med. Res.*, **7**, 238.
Idem, Duncan, J. T., and Henry, T. A.—(1924) *J. Hyg.*, **23**, 1.
Chatterjee, C.—(1908) *Zbl. Bakt.*, 1 Abt., Orig., **48**, 246.
Davis, D. J.—(1917) *J. Infect. Dis.*, **21**, 392.—(1918) *Ibid.*, **23**, 248.
Doryland, C. J. T.—(1916) *J. Bact.*, **1**, 135.

EIJKMAN, C.—(1904) *Zbl. Bakt.*, 1 Abt., Orig., **37**, 436.—(1906) *Ibid.*, **41**, 367, 471.
ELLIS, C., AND MACLEOD, A.—(1923) 'Vital Factors of Food: Vitamines and Nutrition,' pp. 90 *et seq.* London (Chapman and Hall, Ltd.)
FLACK, M.—(1916) *Brit. Med. J.*, **2**, 682.
FUNK, C.—(1922) 'The Vitamines' (2nd Edition), pp. 194, 224, 235. Baltimore (Wilkins and Wilkins, Co.)
HUNTOON, F. M.—(1918) *J. Infect. Dis.*, **23**, 113.
LLOYD, D. J.—(1916) *J. Path. Bact.*, **21**, 113.
MCLEOD, J. W., AND GOVENLOCK, P.—(1921) *Lancet*, i, 900.
PACINI, J. P., AND RUSSELL, D. W.—(1918) *J. Biol. Chem.*, **34**, 43.
PALEVICI, W. S.—(1927) *Giorn. Bat. Immunol.*, **2**, 721.
PLIMMER, V. G., AND PLIMMER, R. H. A.—(1922) 'Vitamines and the Choice of Food,' pp. 62 *et seq.* London (Longmans Green and Co.)
SHEARER, C.—(1917) *Lancet*, i, 59, and ii, 714.
THJÖTTA, TH.—(1921) *J. Exp. Med.*, **33**, 770
Idem, AND AVERY, V. T.—(1921) *Ibid.*, **34**, 97.
WAKSMAN, S. A.—(1920) *J. Bact.*, **5**, 1.
WERKMAN, C. H.—(1927) *Ibid.*, **14**, 335.
WILLIMOT, S. G.—(1928) *Biochem. J.*, **22**, 67.
WINSLOW, C. F. A., KLIGLER, I. J., AND ROTHBERG, W.—(1919) *J. Bact.*, **4**, 429.

TITRATION OF SCARLET FEVER ANTITOXIN.*

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THE only official unit of scarlet fever antitoxin known to the authors is that fixed in the United States (McCoy, 1928; Dyer, 1928). A dried standard scarlet fever antitoxic serum is kept at the Hygienic Bureau, Washington, and the unit is defined as ten times the amount which, in the skin-neutralization test, neutralizes the "test-dose" of toxin. This test-dose contains five "skin test-doses," and one skin test-dose is defined as the amount which gives in the majority of susceptible people a reaction 10 mm. or more in diameter. It is perhaps a little unfortunate that this term "skin test-dose" has crept into the literature. To avoid the confusion between "the test-dose" of toxin and the "skin test-dose" (which is one-fifth of the former), it might be preferable to use instead of "skin test-dose" some such term as "minimal skin dose" (m.s.d.), or "minimal reacting dose" (m.r.d.), as is already current in connection with the intradermic injection of diphtheria toxin.

* The substance of this paper was presented as a report to the Standards Committee of the League of Nations at a meeting at Frankfurt in April, 1928.

Five methods of titrating scarlet fever antitoxin are available:

(A) On the human being:

(1) Schultz-Charlton test—comparing, on the same scarlet fever patients with early rashes, the dilutions of different sera which give positive blanching.

(2) Passive immunity test—giving fixed doses of different sera to a series of Dick-positive patients, and finding how long the patients remain Dick-negative.

(3) Skin-neutralization test with toxin-antitoxin mixtures.

(B) On animals:

(4) Parish-Okell rabbit-test—finding the quantity of antitoxin which will neutralize a culture of streptococcus given intravenously.

(5) Wadsworth-Kirkbride skin-test with goats (1926). Dr. Wadsworth, we understand, still uses this test, but it has not apparently gained many adherents. We ourselves have been quite unsuccessful in getting consistent results, possibly owing to lack of the special goats used at Albany.

Before considering which of these tests will eventually establish itself we must know (1) the error of each test, (2) the practicability of it. With regard to the latter, if an animal test is as accurate as the "human" tests it will presumably eventually supersede them.

ERRORS OF THE TESTS.

There are two obvious methods of investigating these.

(A) A group of sera is sent to different investigators, who are asked to assign unit values to the sera. The results are compared at the end of the investigation, and if they do not agree, the whole test is repeated. We are strongly of opinion that in order to obviate unconscious bias, it is desirable that the person doing the tests should have no indication of the identity of the sera.

(B) A serum is chosen, and from it dilutions of different values, *e.g.* 50, 25 and 12·5 %, are made with non-antitoxic horse-serum or saline solution. These, without any indication of their values, are handed to the various investigators, who are asked to differentiate the sera supplied and determine the values.

Schultz-Charlton Method (Blake and Trask, 1925).

We attach the results of a few illustrative experiments. In an early experiment 31 successive scarlet fever patients were observed; 9 gave no clear blanching at any of the dilutions tried, 22 were therefore left. On these 86 tests were made. Into the rash of each patient were injected 0·2 c.c. of one or more corresponding dilutions of two sera; *e.g.* into the first patient were injected 1/1000 and 1/12,000 dilutions of each of the two sera under examination, A and B. From Table I it will be seen that serum A produced a greater number of blanchings at dilutions 1000, 12,000, 16,000, 24,000 and 36,000. In two instances a dilution of 1/16,000 failed to blanch, whereas 1/24,000 blanched, and on 1 patient 1/1000 of a serum failed, whereas 1/12,000 blanched. There are thus errors of technique or inconsistencies, but on a review of the results

it is reasonable to conclude that serum A is of somewhat greater value than serum B. Other tests than the Schultz-Charlton revealed this same relative potency, but this fact was not known to the investigator who carried out the tests here described.

TABLE I.—*Schultz-Charlton Test, Sera A and B.*

Dilution.	Number of patients.	Number positive.	
		A.	B.
1/1000	10	7	6
1/4000	4	4	4
1/8000	4	3	3
1/12,000	5	1	0
1/16,000	6	4	1
1/24,000	10	4	3
1/36,000	4	2	0

The results suggest that the method is not a very good one, and that in order to differentiate sera a great many patients and tests are necessary.

In Table II are given the results of 256 tests; here it was often not possible to make injections of pairs of corresponding dilutions into the same patient. It is difficult to decide from these results what difference there is in the potency of the two sera, though A appears to be stronger than B.

TABLE II.—*Schultz-Charlton Test, Sera A and B.*

Dilution.	Number of tests.		Number positive.		Per cent. positive.	
	A.	B.	A.	B.	A.	B.
1/1000	35	14	28	6	80	43
1/4000	31	6	22	5	71	84
1/8000	53	10	28	6	53	60
1/12,000	8	8	1	0	12	0
1/16,000	32	8	8	1	25	12
1/24,000	21	15	7	4	33	27
1/36,000	9	6	3	0	33	0
	189	67	97	22		

Table III records another series of observations by a different worker. Here the sizes of the blanched areas were measured. It is fairly clear that on this basis serum C was better than A, for of 36 tests, 20 with serum A gave blanching of less than 10 mm. (which is almost a negative result), whereas with C only 7 went into this class. Serum C consistently produced larger areas of blanching than A. Here again it is obvious that to compare quantitatively two sera a large number of tests are necessary on a considerable number of patients.

In another series of observations seven injections of 0·2 c.c. of various dilutions—some in duplicate in two areas—were given. 1/4000 produced a

blanched area of 40 × 22 mm., 1/8000 produced in one area 5 × 10 mm., in the other 10 × 10 mm., 1/16,000 gave faint 5 × 5 mm. and 15 × 20 mm., 1/32,000 gave *nil* and 5 × 5 mm. Some of these discrepancies were due to variation in the intensity of the rash in the various areas chosen for the injections.

TABLE III.—*Schultz-Charlton Test.*

Diameter of blanched area (mm.).	Serum C.			Total positive.	Serum A.			Total positive.
	1/1000.	1/8000.	1/16,000.		1/1000.	1/8000.	1/16,000.	
0–10	0	1	6	7	0	12	8	20
10–20	3	12	2	17	5	4	0	9
20–30	5	3	0	8	6	0	0	6
>30	4	0	0	4	1	0	0	1
Number of patients	12	16	8	36	12	16	8	36

Table IV records the measurements of the blanched areas produced by four dilutions of a certain concentrated serum tested under favourable conditions. The worker had had a good deal of experience of the test, a particularly vivid early scarlet fever rash was available, and relatively flat areas on abdomen and the inside of the thigh were chosen for the injections. The grading is regular, and a result of this kind suggests that the method could be used as a comparative one. Unfortunately a very little further experience revealed one

TABLE IV.—*Schultz-Charlton Test.*

Dilution of serum.	0·2 c.c. injected.			
	16 hours (mm.).	24 hours (mm.).	40 hours (mm.).	
1/4000	40 × 25	45 × 20	40 × 20	
1/8000	20 × 25	30 × 20	15 × 15	faint.
1/16,000	20 × 20	15 × 20	10 × 10	,,
1/32,000	15 × 15	10 × 10	*Nil*	

difficulty: the giving of a dose of therapeutic serum shortly after the intradermic injections causes such universal fading of the rash that the Schultz-Charlton areas next morning are faint and indefinite and it is difficult or impossible to make satisfactory readings. It is obviously not possible to make a series of observations to find how long the physician must withhold serum to allow the blanching to be satisfactory; the obligation to give serum at once makes this method of testing the value of different sera an impracticable one. But for this objection the clinician could easily test the value of the serum he is using, by injecting various dilutions intradermally until experience had taught him that a serum which had not blanched a reasonable percentage of rashes in a dilution of, *e.g.*, 1/8000 or possibly 1/16,000 was not of satisfactory antitoxic content.

Conclusion.—The Schultz-Charlton method could probably be used as a rough method of titration, if great care were taken in the choice of rash and of the areas injected.

Passive Immunity Method (O'Brien, 1926).

We at first thought it might be possible to compare sera by finding the number of Dick-positive patients who became negative in 24 hours after the injection of corresponding doses of different sera. The results dealing with 216 patients are not promising (Table V). Thus with serum A whereas 24 of

TABLE V.—*Passive Immunity Test.*

Patients Dick negative at 24 hours after various doses of six concentrated antitoxins.

Serum.	Dose of serum in c.c.				
	10.	5.	4.	3.	2·5.
A	1/1	24/27	1/1	8/9	27/30
B	...	14/23	1/1	5/5	24/45
C	...	...	...	...	13/13
D	...	7/7	...	...	3/3
E	...	9/13	...	12/14	10/17
F	...	...	...	...	7/7

27 became negative after a dose of 5 c.c., 27 of 30 became negative with 2·5 c.c. In order to obtain clear results it is necessary to repeat the Dick test on various days to determine how long the patients remain Dick-negative.

TABLE VI.—*Passive Immunity Test.*

Duration of immunity with (*a*) 2·5 c.c., and (*b*) 5 c.c. antitoxin.

1. *Antitoxin D.*

(*a*) 2·5 c.c.			(*b*) 5 c.c.		
No. of patients.	Days after injection.	Dick positive. Dick negative.	No. of patients.	Days after injection.	Dick positive. Dick negative.
16	1	1 15 (94)	8	1	0 8 (100)
	5	2 14 (88)		5	0 8 (100)
	8	11 5 (31)		9	3 5 (62)
	12	16 0 (0)		13	6 2 (25)
				17	7 1 (12)

2. *Antitoxin A.*

16	1	0 16 (100)	14	1	0 14 (100)
	5	4 12 (75)		5	0 14 (100)
	8	14 2 (12)		9	4 10 (71)
	12	16 0 (0)		13	11 3 (21)
				17	14 0 (0)

Table VI records observations by Dr. R. Cruickshank. Consideration of the percentage results shows that (1) no clear differences are obvious from the 24-hour test alone; (2) a dose of 5 c.c. gives better results than 2·5 c.c., *i. e.*

a serum of value x can be distinguished from one of value $2x$ (*vide* particularly 8- and 12-day readings); (3) whether doses of 2·5 c.c. of the two sera D and A be compared, or doses of 5 c.c., these two sera are indistinguishable in value. One cannot therefore be double the value of the other; (4) in order to compare two sera it is necessary to have a considerable number of patients under observation for 10 to 14 days.

We have a record of 130 further observations of this kind which justify the conclusions given above, and similarly emphasize the value of the readings made from 7 to 14 days.

Conclusion.—Given a sufficient number of patients, *e.g.* 10 to 20 under observation for 12 to 14 days, it is possible to distinguish a serum of value x from one of $2x$.

Skin Neutralization Method (Dick and Dick, 1924 and 1925).

Our first attempts to use this method were made on ourselves and some volunteers amongst the staff. We found the greatest difficulty in drawing conclusions and were not certain that we could differentiate two sera of widely different values, or that we could always distinguish between two dilutions, of which one was five times stronger than the other. A good deal of work was done later in conjunction with several clinicians. We give a few tables illustrative of the general results. Table VII records two series of tests by R. Cruickshank (1928). These results suggest that without careful study and a number of patients, it may be impossible to distinguish between dilutions of

TABLE VII.—*Skin Neutralization Test.*

Experiment A.

Dilution of antitoxin.	Area of reaction in mm. S.A. 4627. 24 hours.	S.A. 4627. 48 hours.	S.A. 4565. 24 hours.	S.A. 4565. 48 hours.	Toxin control, 24 hours.
1/1000	*26 × 31	30 × 33	16 × 20	25 × 25	32 × 40
1/800	*17 × 23	17 × 17	14 × 17	17 × 19	21 × 26
1/400	*15 × 23	18 × 23	10 × 13	15 × 21	24 × 27
1/200	*14 × 17	22 × 25	11 × 16	17 × 17	34 × 37
1/100	...	†15 × 15	†20 × 22	†20 × 25	27 × 33

* Each of these figures represents the average of four separate reactions on four children.
† With 1/100 dilution of antitoxin, only one case showed any reaction with both S.A. 4627 and S.A. 4565.

Experiment B.

1/800	20 × 14	22 × 15	15 × 10	17 × 11	...
1/200	19 × 12	19 × 13	15 × 9	17 × 10	...
1/100	...	‡12 × 9	...	...	...

The Table in *Experiment B* gives the average size of the reactions resulting from the injection of dilutions 1/800, 1/200 and 1/100 of each antiserum + equal quantity of toxin, carried out on three children, all aged 12 years.
‡ Two of the cases showed slight reaction with 1/100 of S.A. 4627 at 48 hours.

1/200 and 1/800. In passing, we may note that McCoy (1928) states that only 15% of adults are suitable for the test and a somewhat larger number of children. In our experience from 30% to 50% of children give sufficient reaction to the intradermic injection of serum alone to make the readings difficult after injection of toxin-antitoxin mixtures.

TABLE VIII.—*Skin Neutralization Test.*

1/300 toxin (T.H.A.I.) used throughout.

U.S.A. antitoxin.

Observer.	A.	B.	C.	D.	Total.	Percentage.
1/40	8/8	8/9	5/7	6/6	27/30	90
1/80	8/8	7/8	2/7	6/6	23/29	79
1/160	4/8	0/8	2/7	2/6	8/29	28

8/8 means 8 negative reactions in 8 patients.

Table VIII records the findings of four different observers. The antitoxin used was a sample of an early standard of the Hygienic Bureau, Washington, kindly sent to us by Dr. McCoy; 1/400 c.c. of this serum in Dr. McCoy's hands usually neutralized the test dose of toxin. A toxin was dried by the late Dr. G. R. James in the Standards Department of the National Institute for Medical Research. 0·1 c.c. of 1/300 dilution of the reconstituted toxin was neutralized in most experiments by 0·1 c.c. of 1/400 Washington standard antitoxin. The four observers, A, B, C and D, each had from six to nine suitable subjects available. A reaction of less than 10 mm. at the site of the injection of the 0·2 c.c. of the mixture of toxin and antitoxin was counted as negative. The readings were made at 24 and 48 hours. The "blocking" of the skin reaction must hold for 48 hours. The results are moderately consistent. It will be seen that a group of about 30 subjects would be necessary in order to distinguish a series of dilutions of sera each double the value of the last. A consideration of the results does not suggest that by this method these observers could have always distinguished two sera of values x and $1{\cdot}5x$.

In a recent experiment we Dick-tested 27 subjects. Of these 16 were positive and 11 negative to the test; 7 of the former group and 5 of the latter gave a reaction to 0·2 c.c. of 1/40 dilution of normal horse-serum; 9 were thus available for the test. Three of these were tested in an attempt to use 0·1 c.c. of 1/100 toxin, *i.e.* three test doses; the results were unsatisfactory. Six were tested with mixtures containing 0·1 c.c. of 1/300 toxin, *i.e.* one test dose, mixed with the various antitoxin dilutions. The observer had been given a series of bottles, the values of which were completely unknown to him; two bottles each of dilutions 1/40, 1/80 and 1/160 and one bottle of dilution 1/320 were included in the series, all labelled so as to give no indication of identity. On 4 of the 6 subjects the dilutions were reported on by the observer and placed in correct "order of merit," though it was not easy to make a decision in some instances until all four had been read. In the readings of the remaining two subjects the following errors appeared: 1/80 was returned as greater than 1/40, 1/80 as equal to 1/160, and 1/320 greater

than 1/80. The observer, after a careful review of all his results, placed every bottle in the correct order of value.

Dr. Dyer (1928) gives some interesting tables in his paper showing that the determination of potency made in two different laboratories agreed. But one cannot from these tables estimate the error of the test; we have found in our own work that for this the conditions (A) and (B) set out on p. 84 are necessary. We have been unable to find any publication giving a clear account of tests of this kind in America, where great experience of this method has been gained, and we hope that some may be published.

Conclusion.—Under favourable conditions it appears possible to distinguish sera of values x and $2x$.

Parish-Okell Rabbit Test (Parish and Okell, 1927 [1,2]; 1928).

The usual method of titrating sera in rabbits is as follows: Three rabbits are injected with 0·25 c.c. and three with 0·1 c.c. of a serum which was selected as the laboratory standard about two years ago and which had previously been shown to be of relatively high value on clinical tests. Four rabbits receive 0·25 c.c. of each of the concentrated sera under test, or 1·0 c.c. if the sera are unconcentrated. All the injections are made intravenously, the serum being given four to six hours before the intravenous injection of 5 c.c. of a virulent broth culture. Four rabbits are injected with culture alone as controls. All the rabbits must be in good condition at the time of injection, and of approximately 4 lb. in weight. They are kept under observation for six days, and the majority of rabbits which have received an adequate dose of serum remain alive and in good health until at least three, and nearly always all four, of the controls are dead.

In one series of tests, 80% of 150 rabbits injected with culture alone died within 48 hours, and 87% within 3 days; 86% of 110 rabbits which had previously received an intravenous injection of 0·25 c.c. of standard serum and 59% of 100 rabbits which had received 0·1 c.c. lived for longer than 3 days. 80% of the rabbits which had received 0·25 c.c. and 46% of the rabbits which had received 0·1 c.c. lived for longer than 6 days.

The method is a practicable one, which serves to distinguish good therapeutic sera from those which are of medium or low potency. When an experimental serum has been found to be of low potency by methods of estimation using human beings, or has been considered unsatisfactory in therapeutic use, it has always been possible to obtain confirmation by the rabbit test. When the same sera have been titrated at intervals of several months they have always given consistent results.

As the rabbit test has been on a routine basis in these laboratories for over two years it is obviously of importance to investigate what differences in antitoxic content can be detected. For this purpose dilutions of the standard serum have been made in concentrated diphtheria antitoxin. Four experiments have been recently carried out, each on 48 animals (Table IX). The culture dose varied from 2·5 to 10 c.c. and the period of incubation of the culture prior to injection from 9 to 20 hours in the various experiments. The period of survival of the rabbits is shown in the table, and it will be seen that

in most instances when using eight rabbits, four in each group, we could distinguish serum of value x from that of value $2x$. An erroneous deduction on one test might have occasionally been made, especially when 10 c.c. was the culture dose. It should be noted, however, that for routine purposes 5 c.c. of a 9 to 12 hours' broth culture is the dose usually employed and has given reasonably consistent results.

TABLE IX.—*Rabbit Test.*

Rabbits injected intravenously with serum (Laboratory Standard) 4 to 6 hours before intravenous injection of culture (S.F. 1.)

Test.	Serum dose in c.c.	Culture dose in c.c. 2·5	5	10
A	0·025	– 4 3 1	... ...	... ...
	0·05	– – 6 3	3 3 2 1	... ...
	0·1	– – – –	– 4 2 1	5 3 1 1
	0·25	– – – –	– – – –	– – 5 2
	0·5	... ...	– – – 1	– 4 2 1
	1·0	... ...	... ...	– – 6 4
B	0·025	– 1 1 1	... ...	... ...
	0·05	4 1 1 1	1 1 1 1	... ...
	0·1	2 2 1 1	2 2 1 1	– 3 2 1
	0·25	– – – 2	– 3 2 2	– – 2 2
	0·5	... ...	– – 3 3	– – – 2
	1·0	... ...	... ...	– – 2 1
C	0·025	– 3 1 1	... ...	... ...
	0·05	5 2 2 1	4 1 1 1	... ...
	0·1	– 3 2 2	– – 3 1	5 2 2 1
	0·25	– – – –	– – 2 2	4 3 1 1
	0·5	... ...	– – – 4	– 4 2 1
	1·0	... ...	... ...	– – – 3
D	0·025	– 2 1 1	... ...	... ...
	0·05	– – 6 2	– 6 1 1	... ...
	0·1	– – – –	– – – 3	– 6 4 1
	0·25	– – – –	– – – –	– – 3 2
	0·5	... ...	– – – –	– – – –
	1·0	... ...	... ...	– – – –

– = survived 6 days; 6 = died 6th day, etc.
All serum dilutions made in concentrated diphtheria antitoxin.

It is economical to test six or more sera at one time. By the usual method of testing 10 unknown sera can be titrated on 50 rabbits; each of the unknown sera is injected into 4 rabbits; 6 rabbits are injected with the standard serum prior to culture, and 4 receive the culture dose only. As the error of the test varies directly with the number of rabbits employed, we have occasionally

titrated samples of large bulks of serum on 6 or 8 rabbits, half the animals receiving 0·25 c.c. and half 0·1 c.c. of each serum under test. Where large volumes of serum are concerned a 6 or 8 rabbit test for each sample is still a practicable one.

Note I.—Henry (1925) purified toxin by alcohol precipitation. Since the completion of this report Pulvertaft has published a somewhat similar method, and Hartley has by dialysis and precipitation concentrated toxin. With toxins prepared by Hartley's method we have been able to kill rabbits by an intravenous injection of 0·1 c.c. to 0·5 c.c. It appears possible to prepare continuous supplies of stable specific toxin which can be used for accurate titration of antitoxin. This modification of the rabbit test may become the accepted method of standardization.

Note II.—Dyer (1925) attempted to titrate scarlet fever antitoxin by the flocculation method. We understand that his later results do not suggest that the flocculation value was a measure of therapeutic efficiency. Kanyo (personal communication) also tried this method, and Ramon (1928) has recently stated that he considers the flocculation results indicate the true antitoxic content. We have tried this method at different periods during the past five years, and though able to produce precipitates with various sera, we have hitherto been unsuccessful in obtaining agreement between these values and the true antitoxic values of sera.

SUMMARY AND CONCLUSIONS.

(1) The Schultz-Charlton test is an unsatisfactory method of titrating scarlet fever antitoxin. It is doubtful if sera of values x and $2x$ can be distinguished by this method on any practicable number of patients.

(2) By the passive immunity method one can distinguish serum of value x from value $2x$ if from 10 to 20 human patients are available for 12 to 14 days.

(3) Skin neutralization test: The error of this method does not seem to be accurately known. Under favourable circumstances and with a considerable number of subjects available, it appears possible to distinguish a serum of value x from one of value $2x$.

(4) By the rabbit test two sera of values x and $2x$ can be usually distinguished with 8 rabbits. The method has been in routine use for over two years and has proved more useful in practice than any of the foregoing tests. With a small number of rabbits results have been obtained which compare favourably in accuracy with those obtained by any of the methods which involve the use of human beings.

In this work we have had the co-operation of a number of clinical workers, including Drs. W. T. Benson, R. Cruickshank, E. H. R. Harries, E. James, A. Joe, W. M. MacFarlane, J. Reid, C. Rundle, J. Smith and F. Thomson. We are indebted to several for the opportunity of making a considerable number of observations and tests ourselves.

REFERENCES.

BLAKE, F. G., AND TRASK, J. D.—(1925) *Boston Med. Surg. J.*, **193**, 659.
CRUICKSHANK, R.—(1928) *Lancet*, **2**, 274.
DICK, G. F., AND DICK, G. H.—(1924) *J. Amer. Med. Assoc.*, **82**, 265, 1246.—(1925) *Ibid.*, **84**, 802.
DYER, R. E.—(1925) *Publ. Hlth. Rep. Wash.*, **40**, 865.—(1928) *Ibid.*, **43**, 1659.
HARTLEY, P.—(1928) *Brit. J. Exp. Path.*, **9**, 259.
HENRY, H.—(1925) *Lancet*, **1**, 710.
MCCOY, G. W.—(1928) 'The Newer Knowledge of Bacteriology and Immunology,' E. O. Jorden and I. S. Falk. Chicago (University Press), p. 955.
O'BRIEN, R. A.—(1926) *Med. Officer*, **25**, 143.
PARISH, H. J., AND OKELL, C. C.—(1927[1]) *Lancet*, **1**, 71.—(1927[2]) *J. Path. Bact.*, **30**, 521.—(1928) *Lancet*, **1**, 746.
PULVERTAFT, R. J. V.—(1928) *Brit. J. Exp. Path.*, **9**, 276.
RAMON, G., MARTIN, R., AND LAFAILLE, A.—(1928) *C. R. Soc. Biol. Paris*, **99**, 734.
WADSWORTH, A. B.—(1927) 'Standard Methods.' London (Baillière, Tindall & Cox), p. 330.

THE DISTRIBUTION OF VIRUS AND NEUTRALIZING ANTIBODIES IN THE BLOOD AND PATHOLOGICAL EXUDATES OF RABBITS INFECTED WITH VACCINIA.

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THE results of different workers who have attempted to demonstrate the presence of vaccinia virus in the blood-stream of infected rabbits have shown irregularities hitherto inexplicable. Calmette and Guerin (1901) reported that they never recovered virus from either the blood or defibrinated blood by application of the material to the shaved skin. Rivers and Tillett (1923) withdrew heart-blood from rabbits four days after skin-scarification with virus and injected it intravenously into normal rabbits which had been shaved and scarified, this led to vaccinial lesions on the irritated skin areas followed by immunity. Levaditi and Nicolau (1923) tested blood at intervals from the 9th day following infection, but never recovered virus. Hoen, Tschertkow and Zipp (1926) attempted blockade of the reticulo-endothelial system in the hope of preventing disappearance of virus from the blood-stream, but even so were only able to recover it on the 4th and 5th day, and Goldman (1928) and Walthard (1927), who used the same technique, were no more successful. Ohtawara (1922), however, by using intra-testicular inoculations as the method of test, obtained reactions fairly regularly with blood samples taken from the

1st to the 10th day of the disease. His work was confirmed later by Gildemeister and Heuer (1928).

Andrewes (1928) found that the intra-testicular inoculation of a mixture of virus with its specific antiserum might give rise to a vaccinial orchitis, even although the same mixture was entirely innocuous when injected intradermally. This phenomenon affords a possible explanation of the more constant recovery of the virus by Ohtawara as compared with other workers, and suggests that many of the negative results might have been due, not to the disappearance of virus from the circulation, but to the simultaneous presence of both virus and neutralizing antibodies in the blood. Many facts support the view that vaccinial antibodies are not directly viricidal, indeed active virus has been found by Douglas, Smith and Price (1929) in certain tissues of a rabbit as long as 41 days after massive infection, at a time when the blood-serum probably contained a very high antibody content.

I. THE DEMONSTRATION OF VIRUS IN THE BLOOD OF INFECTED RABBITS.

1. *Fixation of Virus by the Formed Elements of the Blood.*

It was decided to fractionate the blood of infected rabbits and to test the fractions as well as the whole blood separately for evidence of virus.

(i) *Technique.*

Strain of virus.—The strain of vaccinia virus used throughout these experiments was a neurovaccine obtained in the first place from Dr. Levaditi. It was propagated for about three years by serial intra-cerebral inoculations in rabbits after which it was transmitted from testis to testis of the same species for several months.

Mode of infection.—Two routes of infection were employed: (*a*) Intravenous inoculation; (*b*) intradermal inoculation. Rabbits inoculated intravenously with one exception, received from 1 to 3 c.c. of an emulsion of vaccinial testis (1 part testis to 50 parts saline approximately) into the marginal ear-vein; such inoculation almost invariably produces a widespread generalized disease (Douglas, Smith and Price, 1929). The emulsions were either allowed to sediment for half-an-hour or lightly centrifuged before use. The animals inoculated intradermally received 6 or 8 inoculations each of 0·2 c.c. testicular emulsion simultaneously into the epilated skin of the back.

Withdrawal of samples.—Nine volumes of blood were allowed to drop directly from an incision in the marginal ear-vein of the infected rabbit into a sterile tube containing 1 volume of an anticoagulant. In the experiments where platelets were required, the anticoagulant was sodium citrate solution 15%, in all other cases it was heparin solution 2 gm. per litre.

Fractionation.—The separation of plasma from the blood-cells was effected by centrifuging 4 c.c. of heparinized blood at about 3000 revolutions per minute for 15 minutes. The supernatant plasma was transferred to a sterile tube by means of a capillary pipette and replaced by saline. After thorough mixing of the cells and saline the suspension was again centrifuged and the supernatant fluid discarded. The cells were washed in this way at least three times after which they were re-suspended in sterile saline to the original volume.

Test inoculations.—All fluids and suspensions were tested for the presence of virus by inoculation of 0·2 c.c. intradermally into the epilated skin of the back of a normal rabbit. The inoculations were widely spaced. Results were read on the 3rd, 4th and 5th days.

Immunity tests.—Intra-cerebral inoculation of highly virulent brain-emulsion was used as the test method, and survival for ten days without the appearance of symptoms was accepted as evidence of solid immunity.

(ii) *Infectivity of Blood after Intravenous Inoculation.*

Preliminary experiments were carried out in order to test the validity of the hypothesis that virus may be demonstrable in some fraction of the blood whilst being entirely masked in the whole blood.

Samples of blood were withdrawn from two rabbits, Nos. 779 and 786. Rabbit 779 had received, 7 days previously, an intravenous inoculation of 3 c.c. of peritoneal exudate from an animal suffering from vaccinial peritonitis. On the day of bleeding pocks had appeared in the mucous membrane of nose and in the hairy skin. Rabbit 786 had received an intravenous inoculation of vaccinical testicular emulsion 4 days previously, it also showed the first signs of generalization of the disease on the day the blood sample was obtained. Both blood samples were fractionated into plasma and washed cells. The fractions and also the whole blood were tested for the presence of virus by the intradermal inoculation of 0·2 c.c. into the epilated skin of a normal rabbit, No. 788.

The result was read on the 4th day after inoculation. The whole blood of Rabbit 786 produced a very slight reaction consisting of a single small pock at the point of inoculation which was only just appreciable when the skin was pinched up between the finger and thumb. The washed-cell suspension, however, gave a very strong reaction, a red inflammatory swelling about 1·5 c.m. in diameter, hard and tender to the touch. The plasma gave no sign of reaction whatsoever. In the case of the 7th day sample from Rabbit 779 there was no sign of reaction from either the whole blood or plasma, but the washed-cell suspension gave rise to an inflammatory swelling which was quite easily seen and felt.

Blood was again withdrawn from Rabbit 779 on the 13th day after infection and tested in an exactly similar manner. In this case all fractions gave negative results.

TABLE I.—*Infectivity of Blood after Intravenous Inoculation: Preliminary Experiments.*

Source of blood. Rabbit No.	Day of bleeding after injection.	Result of test inoculation of blood fractions.			Test rabbit.	
		Whole blood.	Plasma.	Washed cells.	No.	Result of immunization test.
786	4	±	—	++	788	Immune.
779	7	—	—	+	788	„
779	13	—	—	—	798	Immune from other inoculations.

The test rabbits were subsequently tested for immunity, they showed no symptoms after intra-cerebral inoculation of highly virulent vaccinial brain emulsion.

The results of these preliminary experiments are summarized in Table I.

A series of experiments was now carried out with blood samples from two rabbits in an attempt to elucidate the following questions:

(1) Is it possible to recover vaccinia virus from blood-plasma at any stage of the infection?

(2) How long after intravenous inoculation does the blood continue to harbour virus demonstrable by the fractionation technique?

Protocol 1.—Rabbit 796 was inoculated intravenously with 3 c.c. of vaccinial testis emulsion, 4½ c.c. of blood was withdrawn exactly 30 minutes after the inoculation, fractionated and tested by intradermal inoculation in a normal rabbit. Twenty-four hours later a second sample of blood was withdrawn and tested, and thereafter daily samples up to the 5th day inclusive. The first sign of generalization of the disease appeared on the 4th day as a pock on the lower lip. On the 5th day the animal became very ill. There was a muco-purulent discharge from the eyes and nose, the respiration was rapid and laboured, and several pocks showed clearly around the mouth and nose. An attempt at bleeding from the ear-vein in the usual manner had to be abandoned. The rabbit was therefore killed and the sample obtained by cardiac puncture immediately after death.

Protocol 2.—Rabbit 797 was inoculated intravenously with 2 c.c. vaccinial testis emulsion. Daily blood samples were obtained and tested from the 1st to the 10th day after infection. The first pocks appeared on the 5th day, a widespread eruption was evident on the 7th day. The rabbit, although obviously ill during this period of eruption, entirely recovered, and was used again later as an immune animal.

Up to the 5th day on which Rabbit 796 was killed the daily samples from both animals were inoculated into the same test animal; those from Rabbit 796 into the skin of one flank, those from Rabbit 797 into corresponding points in the skin of the opposite side. The experiment was planned in duplicate in this way, firstly as a precaution against a fatal outcome of the infection, and secondly so that any differences found in the inoculation results from the two cases should not be ascribed to differences in susceptibility of test rabbits. Such a precaution was evidently necessary because of the irregularity in the results of whole blood inoculation which did not run exactly parallel in the two cases.

It will be seen from Table II that virus was demonstrated in the plasma up to 24 hours after infection, but had apparently disappeared in 48 hours and thereafter never reappeared. The results given by whole blood inoculation were irregular in both cases, thus confirming previous work carried out in conjunction with Douglas and Price. The washed-cell suspensions, however, gave definite reactions regularly, up to the day of death in one case, up to the 8th day in the other case.

A repetition of this series of experiments was carried out.

Protocol 3.—Rabbit 934 was inoculated intravenously with 2 c.c. vaccinial testis emulsion. The first blood sample was withdrawn 24 hours after infection, thereafter samples were obtained daily up to the 10th day. In each case

TABLE II.

Time of bleeding after infection.	Results of test inoculations of blood fractions.						Test rabbits.	
	Rabbit No. 796.			Rabbit No. 797.			No.	Result of immunization test.
	Whole blood.	Plasma.	Washed cells.	Whole blood.	Plasma.	Washed cells.		
½ hour	++	+	++	Not tested			798	Immune.
1 day	++	+	++	++	+	++	799	„
2 days	+	−	++	−	−	++	804	„
3 „	+	−	++	+	−	++	805	„
4 „	+	−	++	−	−	++	806	„
5 „	−	−	+	−	−	+	807	Died under anæsthetic.
6 „	...	...	...	−	−	+	808	Immune.
7 „	...	...	...	−	−	+	809	„
8 „	...	...	...	−	−	+	810	„
9 „	...	...	...	−	−	−	811	Not immune.
10 „	...	...	...	−	−	?	816	Immune.

whole blood, washed cells, and plasma were tested for the presence of virus. The first lesion to appear was a pock on the lower lip on the 4th day after infection, many lesions had developed by the 5th day. The rabbit was very ill at this time, but made a complete recovery. The results shown in Table III are not so regular as those of the preceding series, but again show the value of removing the plasma from the formed elements. No reactions were obtained on the 6th, 7th and 8th days, but on the 9th day virus reappeared in the circulation, and although no reaction was given by whole blood, that given by the washed cells was distinct.

TABLE III.—*Fractionation of Blood Samples from Rabbit 934.*

Day of blood sample.	Test rabbit.	Results of test inoculations.		
		Whole blood.	Plasma.	Washed cells.
1	938	+	−	+
2	941	+	−	++
3	946	+	−	++
4	947	−	−	+
5	948	−	−	+
6	949	−	−	−
7	950	−	−	−
8	951	−	−	−
9	952	−	−	+
10	959	−	−	−

(iii) *Infectivity of Blood after Intradermal Inoculation.*

In the previous experiments the infected animals received very large doses of virus directly into the circulation. It was decided to test the value of the

fractionation technique in recovering virus from the blood of rabbits infected by intradermal inoculation. Two series of experiments were carried out.

Protocol 4.—Rabbit 908 received 6 intradermal inoculations on 19 . 11 . 28 in the epilated skin of the back, each inoculation consisting of 0·2 c.c. Rabbit 883 testis Ringer diffusate. The skin reactions were fairly large by the third day, but never exceeded 2 cm. in diameter, and never showed the central necroses with black eschar which is so characteristic of skin reactions following the intradermal inoculation of vaccinial material of very high virulence. There was also no appearance of a generalized skin eruption and no evident malaise. Blood samples were withdrawn from the 1st to the 10th day, each being fractionated into plasma and washed cells.

Protocol 5.—Rabbit 937 received 8 intradermal inoculations on 5 . 12 . 28, each consisting of 0·2 c.c. Rabbit 883 testis emulsion. The resultant skin lesions were over 3 cm. in diameter and necrotic, the rabbit was very ill on the 4th day and later showed skin generalization. A blood sample was withdrawn ½ hour after the inoculations; thereafter daily samples were tested up to the 10th day.

The results of both series of experiments are shown in Table IV. The value of fractionation is well exemplified, had the whole blood only of Rabbit 908 been tested, the conclusion might have been drawn that no appreciable invasion of the circulation by virus had occurred at any stage of the disease. In the case of Rabbit 937 virus appeared in the blood as early as 24 hours after infection, the washed cells always gave rise to larger reactions than the whole blood, and, in two instances, produced reactions when the results of whole blood inoculation was entirely negative.

TABLE IV.—*Distribution of Virus in Blood after Intradermal Injection.*

Time of sampling after injection.	Rabbit No. 908.			Rabbit No. 937.		
	Whole blood.	Plasma.	Washed cells.	Whole blood.	Plasma	Washed cells.
½ hour	....	....	....	−	−	−
1 day	−	−	−	+	−	++
2 days	−	−	+	+	−	++
3 ,,	−	−	+	+	−	++
4 ,,	−	−	−	±	−	+
5 ,,	−	−	−	−	−	+
6 ,,	−	−	−	−	−	−
7 ,,	−	−	−	−	−	−
8 ,,	−	−	−	−	−	−
9 ,,	−	−	−	−	−	+
10 ,,	−	−	−	−	−	−

2. *Which Type of Cell Fixes the Virus?*

From the foregoing it appears highly probable that when the virus of vaccinia gains access to the circulation of a rabbit it is taken up by the formed elements of the blood. In no less than 15 of the experiments recorded here

virus has been revealed by removing the plasma, although its presence was entirely masked when the whole blood was tested. It may be added that many later experiments carried out with a different object in view have incidentally given confirmatory results.

An attempt was now made to determine whether all the formed elements share in this virus fixation or, if not all, which are responsible.

(i) *Technique.*

Red blood-corpuscles free from white cells were obtained by utilizing the filtration method of Fleming (1926). Ten c.c. of heparinized blood was filtered three times through tightly-packed cotton-wool, the filtrate was centrifuged, the plasma removed, the red cells washed three times and then re-suspended in saline. It was found advantageous to carry out the filtration in the hot room at a temperature of 37° C. and to warm up all the apparatus to this temperature before use. Smears of the filtrate were made and stained with Leishman's stain, apart from an occasional lymphocyte they showed no white cells.

To obtain suspensions of white blood-cells one of the three following methods was used:

(*a*) The buffy coat from a centrifuged washed cell suspension was scooped up in a spoon and re-suspended in saline.

(*b*) The red cells underneath the buffy coat were sucked up in a capillary pipette and discarded. The process was repeated until the cellular deposit after centrifugation showed no differentiated strata.

(*c*) Cellular suspensions were very lightly centrifuged to throw down as far as possible the red cells only, the supernatant layer containing most of the white cells and the platelets was removed and the white cells separated by further centrifugation.

None of these methods was found to be entirely satisfactory, for in all cases the resultant suspensions contained some red corpuscles. It was found possible, however, by any of the methods to obtain suspensions in which at least 90% of the cells were of the white variety.

Blood-platelets were obtained by the differential centrifugation of citrated blood. They were washed three times before re-suspension in saline, the volume of saline used being from a fifth to a tenth that of the original blood sample, in order to offset the unavoidable loss of some of the platelets in the deposit from the first centrifugation. Stained smears were examined, they always showed some white cells, but if too many of these were seen the suspensions were rejected.

(ii) *Experimental.*

Blood was withdrawn from Rabbit 874 which had received an intravenous inoculation of virus three days previously. In addition to the fractions hitherto tested, emulsions of white cells and red cells were obtained. A similar fractionation was carried out on a blood sample from Rabbit 832 infected 4 days previously.

The results, which are shown in Table V, appear to implicate the white

blood-cells in virus fixation and to exclude the red cells. In each case those suspensions containing white cells produced vaccinial reactions, whilst those inocula containing no white cells produced no reactions whatsoever.

TABLE V.—*Fixation of Virus by White Blood-Cells.*

Fractions tested.	Rabbit 874. 3rd-day blood.	Rabbit 832. 4th-day blood.
Whole blood	± (1 small pock)	±
Plasma	−	−
Washed cells (W. and R.)	+	Not tested.
White blood-cells	+	+
Red blood-cells (filtered and washed)	−	−

There remained the possibility of virus adsorption by the blood-platelets, and although several experiments have been carried out with a view to settling the question this possibility has not yet been satisfactorily excluded. It will be seen from Table VI that in all experiments, except one, vaccinial reactions were produced by inoculation of the platelet suspensions. In no case, however, have these suspensions been entirely freed from leucocytes. The white cells produced reactions in all cases, but unfortunately the white cells suspensions always contained some platelets. The reactions produced by the white cells, however, were invariably larger than those produced by the platelets, some of the latter were indeed so small and abortive as to lead to the impression that white blood-cells play the chief, if not the only part in virus fixation.

TABLE VI.—*Infectivity of Blood-Fractions with Special Reference to the Platelets.*

Source of blood. Rabbit No.	Day of bleeding after infection.	Infectivity of blood-fractions. Platelets.	White cells.	Washed cells (W. and R.).	Red cells.	Plasma.	Whole blood.
915	3	+	...	+	...	−	−
„	6	+	...	...	...	...	...
„	7	−	...	+ + +	...	--	+
968	3	+	+	...	−	−	−
„	6	+	+ + +	...	−	±	+
969	3	+	+	...	--	−	−
978	4	+	+ + +	...	...	±	...
979	4	+	+ +	...	...	−	...

(3) *Rapid Disappearance of Virus Inoculated into an Immune Rabbit.*

A rabbit which has recovered from generalized vaccinia possesses a very high grade of immunity against subsequent infection. The intravenous inoculation of large doses of virus into such an animal entirely fails to produce any appreciable reaction, the fate of the virus is unknown. It was thought of

interest to determine whether a fixation of virus by the formed elements of blood would occur in the immune animal, and if so, how long after inoculation the virus could be recovered.

A blood sample was withdrawn from Rabbit 797 on the 31st day after infection when all signs of generalization had disappeared, and the animal appeared in perfect health. The blood was fractionated and tested for virus with entirely negative results. The rabbit was then inoculated intravenously with 2 c.c. testicular emulsion. Blood was withdrawn exactly half an hour after the inoculation, and again 24 hours later. The washed cell suspensions from the half-an-hour specimen produced a specific reaction on intradermal inoculation into a test rabbit. The whole blood and plasma gave no reactions. In the 24-hour specimen no virus could be demonstrated in any fraction.

The experiment was repeated on the same animal, after a further interval of 54 days. The blood was tested before the re-inoculation and found negative. Samples were then withdrawn at intervals of ½, 1, 2, 3, 4, 6 and 8 hours. The results shown in Table VII indicate that virus injected into the blood-stream of an immune rabbit is taken up at least in part by the formed elements and disappears from the circulation in less than 6 hours.

TABLE VII.—*Disappearance of Virus from Blood when Inoculated into an Immune Rabbit.*

Rabbit.	Interval between inoculations.	Time of sample.	Whole blood.	Plasma.	Washed cells.
797	54 days	½ hour	±	−	+
,,	...	1 ,,	−	−	+
,,	...	2 hours	−	−	+
,,	...	4 ,,	−	−	+
,,	...	6 ,,	−	−	−

II. THE RISE OF ANTIBODIES IN BLOOD PLASMA AFTER INFECTION.

The experiments recorded above suggested that vaccinia virus may remain constantly in the blood-stream of infected rabbits for at least 8 days, that it is quickly taken up by or adsorbed on the white blood-cells and that some factor appears in the blood-plasma, in some cases even as early as the second day of the disease which is capable of masking the presence of the virus when the whole blood is tested.

It was, therefore, decided to repeat the fractionation experiments on the blood of inoculated rabbits and to test the plasma obtained from each sample for the presence of neutralizing antibodies.

Protocol 6.—Rabbit 817 and Rabbit 818 were each inoculated intravenously with 2 c.c. of an emulsion of vaccinial testis. From the 2nd to the 5th day, blood was obtained from the ear-vein in the manner previously described. On the 6th day both animals were extremely ill and showed the symptoms and signs of severe generalized vaccinia. They were therefore anæsthetized with chloroform and blood obtained by heart-puncture. They were not allowed to recover from the anæsthetic. Post-mortem examination in both cases showed extensive generalized vaccinial lesions.

The daily blood samples were fractionated immediately after withdrawal and the whole blood and washed cells tested for the presence of virus in the usual manner. The plasma, however, was tested for the presence of neutralizing antibodies as follows. Ten-fold dilutions of a testis Ringer diffusate were made with sterile saline. Half c.c. quantities of each dilution were set out in sterile tubes in duplicate. To each tube of one series $\frac{1}{2}$ c.c. of sterile saline was added; to each tube of the other series $\frac{1}{2}$ c.c. of the plasma to be tested. After thorough mixing 0·2 c.c. of each solution was inoculated intradermally into the epilated skin of the back of a normal rabbit, the inocula containing virus only into one side, those containing virus and plasma into corresponding sites on the opposite side. Reactions were read on the 4th day. A schematic representation of such a test showing the results obtained in one case will help to make this clear:—

Head.

	Left side. Virus dilutions only.	Right side. Virus dilutions with plasma; 1 in 2 dilution.	
1/2000 virus dilution.	−	−	1/2000 virus dilution.
1/200 „	+	−	1/200 „
1/20 „	+ +	+	1/20 „
1/2 „	+ + +	+	1/2 „

− = no reaction; + = a small pock; + + = reaction ¼ in. diameter; + + + = reaction ¼–½ in. diameter.

It is clearly evident that in this case the plasma exerted a considerable neutralizing effect. It is impossible, however, to assess accurately the antibody content of the plasma because a 1/2 dilution of virus when mixed with plasma gave rise to no greater reaction than a 1/20 dilution with the same volume of the same plasma. This phenomenon which is of constant occurrence was pointed out by Andrewes (1928).

The results of this series of experiments are summarized in Table VIII. The figures used to express the neutralizing power of the plasma specimens provide only a rough approximation.

The results shown in Table VIII confirm those of previous fractionation experiments. The demonstration of virus in whole blood was irregular in both cases, whilst the inoculation of a suspension of washed cells produced constant and comparatively large reactions.

The plasma of both rabbits appeared to possess some neutralizing power 48 hours after injection, slight but sufficient to render the inoculation of whole blood entirely innocuous in spite of their being active virus fixed by the cells. There was an apparent increase of antibodies on the 3rd day followed by a decrease on the 4th and 5th days. The decrease coincided with a return of demonstrable virus in whole blood, and therefore suggested that there might be an outpouring of virus from focal lesions into the blood-stream at this stage of the disease, as a preliminary to the production of generalized lesions

throughout the body, such outpouring of virus might possibly account for the using up of antibodies with a corresponding decrease in the amount left free in the plasma. The validity of this interpretation of the results was considered doubtful because a different test rabbit was used for the titration of each day's plasma sample. There is a definite variation in the degree of susceptibility of different rabbits to vaccinia virus.

TABLE VIII.—*Simultaneous Presence of Virus and Antibodies in Blood.*

	Day of bleeding after infection.	Test Rabbit No.	Presence of virus in blood-fractions.		Antibody content of plasma.
			Whole blood.	Washed cells.	Number of M.I.D.* neutralized by 1 c.c.
Rabbit No. 817	2	819	+	++	10–100
	3	821	−	+	100–1000
	4	823	−	+	100–1000
	5	825	±	+	10–100
	6	827	−	+	1000
Rabbit No. 818	2	820	−	++	10–100
	3	822	−	+	100–1000
	4	824	±	+	10
	5	826	±	+	10–100
	6	828	±	+	100–1000

* M.I.D. = Minimal infective dose.

To overcome this difficulty the following experiment was carried out. A rabbit previously infected by intravenous inoculation was bled into heparin solution daily from the 1st to the 7th day. The plasma from each day's sample was stored in the cold room (0° C. to 5° C.) until the final bleeding on the 7th day. An eighth specimen of normal plasma was obtained for use in control inoculations.

Five rows of eight tubes were set out, those in the 1st row each received 0·5 c.c. of 1/10,000 dilution of virus diffusate; those in the 2nd row, 0·5 c.c. of a 1/1000 dilution and so on; the 5th row receiving undiluted diffusate. 0·5 c.c. of normal plasma was now added to the first tube in each row. 0·5 c.c. of 1st-day plasma to the second tube of each row, etc. Thus the final dilutions of virus now ranged from 1/2 to 1/200,000, and every tube contained a 1/2 dilution of some one of the plasma specimens.

Five normal rabbits were epilated to receive the test inoculations; each received eight intradermal inoculations of 0·2 c.c. of the mixtures containing certain virus dilution.

Table IX shows the results of this experiment.

There is one anomalous result given by the mixture of the 4th-day plasma and the 1/2000 dilution virus, which up to the present had remained inexplicable. Apart from this the evidence suggests that antibodies in this case first appeared on the third day, decreased slightly on the fourth and thereafter steadily increased up to the seventh. It may be noted that the first lesions of generalization appeared on the fourth day.

This experiment has been twice repeated, once on the plasma of an

intradermally infected animal. In both cases there was evidence of antibody formation on the 2nd day after infection, but in both cases irregularities in the 3rd-day plasma reactions appeared like that noted above. An adequate explanation of this irregularity on the day following the first appearance of antibodies has not been found. Further investigations are in progress.

TABLE IX.—*Rise of Antibodies in Plasma of Rabbit.*

Test Rabbit No.	Virus dilution.	Control. N.	Day on which plasma was obtained after infection. 1.	2.	3.	4.	5.	6.	7.
853	1/20,000	+	+	+	−	+	−	−	−
854	1/2000	+ +	+ +	+ +	+	−	−	−	−
855	1/200	+ + +	+ + +	+ + +	+ +	+ + +	+ +	±	±
856	1/20	+ + +	+ + +	+ + +	+ +	+ + +	+ +	+	±
857	1/2	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+	±

N.B.—Plasma dilutions in all cases = 1 in 2.

III. SIMULTANEOUS PRESENCE OF VIRUS AND ANTIBODIES IN PATHOLOGICAL EXUDATES.

A condition of vaccinial peritonitis may be produced by the intra-peritoneal, or in some cases the intra-testicular, inoculation of a highly virulent strain of neurovaccine. (Douglas, Smith and Price, 1929). The post-mortem findings in such cases are by no means constant, but vary, especially as regards the amount and nature of the peritoneal exudate which may be scanty or abundant, clear or very turbid, non-viscous or viscous.

On testing a number of exudates for the presence of virus by intradermal inoculation into normal rabbits irregular results were obtained. In some, no virus whatsoever was demonstrated, whilst in others a high dilution was found capable of producing a specific vaccinal reaction. This irregularity suggested that the phenomenon found in the case of blood from infected rabbits might also occur in pathological exudates. It was therefore decided to attempt similar fractionation experiments.

(i) *Technique.*

Three routes of infection were employed, intra-peritoneal, intra-testicular and intravenous. In all cases the inoculum consisted of an emulsion of highly virulent vaccinial testis.

Collection of exudates.—In all experiments the animals were killed and the peritoneal cavity opened as rapidly as possible, consistent with adequate precautions of sterility. The exudate was withdrawn with a pipette which had been washed through with sterile heparin solution and transferred immediately to a tube containing heparin solution.

The formed elements were separated in most cases by centrifugation, the deposit being washed three times before final re-suspension in saline. In one experiment the exudate clotted before this could be accomplished, the clot was therefore removed and emulsified in saline, then centrifuged, washed, and re-suspended as before.

Filtration.—A perfectly clear, supernatant fluid could not be obtained by centrifugation. Portions of the exudates were therefore filtered through Seitz asbestos filters. 30 to 40 mm. mercury negative pressure was necessary for filtration, even so, the filtration time varied greatly with the different specimens.

Test inoculations.—Antibody titrations and immunity tests were carried out in exactly the same manner as in the blood experiments.

(ii) *Experimental.*

Five experiments were carried out, four of them with peritoneal exudates from four different rabbits, the fifth with a pleural exudate from one of the same animals.

Experiment 1.—Rabbit 844 was inoculated with an emulsion of vaccinial testis, each testis being injected with 0·8 c.c. The testes were very swollen on the 3rd day after infection. When the rabbit was killed an abundant turbid peritoneal exudate was obtained. This gave rise to no virus reaction when tested by intradermal inoculation into a normal rabbit, but an emulsion of the clot which formed soon after withdrawal produced clear evidence of virus content. Antibodies were found in the filtrate.

Experiment 2.—Rabbit 852 was inoculated intra-peritoneally with 2 c.c. of vaccinial testicular emulsion. It became very ill on the 3rd day and was killed on the 4th day. An abundant turbid peritoneal exudate was withdrawn. This gave rise to a vaccinial reaction in a dilution of 1/10,000. Washed cells produced a strong reaction and the supernatant fluid after centrifugation for half an hour a slight reaction. This supernatant fluid was not clear. A filtrate showed a high antibody content.

Experiment 3.—Rabbit 852 also gave a pleural exudate. Only the whole exudate and a filtrate were tested; the former showed virus in a dilution of 1/10,000; the latter a high content of antibodies.

Experiment 4.—Rabbit 876 received 1·5 c.c. vaccinial testis emulsion intravenously. Pocks appeared on the 4th day on the mucosæ of lips and nose and also in the hairy skin. On the 6th day the rabbit was very ill, but had recovered by the 11th day when it was killed. A fairly abundant, slightly turbid peritoneal exudate was obtained. This showed no virus, even in the washed cells; a filtrate showed antibodies.

Experiment 5.—Rabbit 886 was inoculated intra-peritoneally with 2 c.c. of vaccinial testis emulsion. It was killed on the 4th day up to which time it had shown no symptoms or signs of disease. A moderate quantity of slightly turbid peritoneal exudate was found. The washed cells gave rise to a vaccinial reaction, the whole fluid proved innocuous. A filtrate possessed no appreciable antibodies.

The results of the experiments are summarized in Table X, and although irregular they indicate quite clearly that both active virus and neutralizing antibodies may exist side by side in pathological exudates due to vaccinial infection. Antibodies were demonstrated in Seitz filtrates in four out of five experiments; in the fifth experiment although not present in a filtrate to a measurable extent one may assume their presence from the fact that virus was masked in the whole fluid, but demonstrable in the washed cell suspension.

TABLE X.

Exudate.	Source of exudate. Rabbit No.	Mode of infection.	Day of death after infection.	Whole fluid.	Washed cells.	Emulsion of clot.	Supernatant fluid.	Filtrate.	Antibody content in filtrate.
Peritoneal.		I.T.							
Abundant, turbid	844	Inoculated with 0·8 c.c. testicular emulsion into each testis	3	—	—	+	...	—	+
Peritoneal.		I.P.							
Abundant, turbid	852	Inoculated with 2 c.c. testicular emulsion	4	+ + +	+ + +	...	+	—	+ + +
Pleural.									
Abundant, turbid	852	Ditto	4	+ + +	...	...	...	—	+ + +
Peritoneal.		I.V.							
Moderate quantity, slightly turbid	876	Inoculated with 1·5 c.c. testicular emulsion	11	—	—	...	...	—	+ +
Peritoneal.		I.P.							
Moderate quantity, slightly turbid	886	Inoculated with 2 c.c. testicular emulsion	4	—	+	...	—	—	—

I.T. = intra-testicular inoculation; I.P. = intra-peritoneal inoculation; I.V. = intravenous inoculation.

In only one case, Rabbit 876 was virus apparently quite absent, a result which is not surprising in view of the fact that the animal was not killed till the 11th day after infection.

DISCUSSION.

Vaccinia virus which gains access to the circulation of a rabbit enters into a close association with some of the formed elements of the blood. Although from the evidence as yet available the platelets cannot be definitely excluded it would appear most probable that the white blood-cells are responsible for this virus fixation, a view which derives support from the fact that a similar process has been demonstrated in the case of peritoneal exudates due to vaccinia which do not contain platelets. It is not known which type of white cell is of importance in this connection or whether all types play a part. A few lymphocytes were usually found in the totally inactive suspensions of red blood-corpuscles, but the number present was so small that they might have contained virus and yet have failed to produce any reaction.

It is of interest that a similar association of virus with white blood-cells has been found in the case of cattle plague by Todd and White (1914) and by Daubney (1928), in human typhus by Nicolle, Conor and Conseil (1912) and in fowl plague by Todd (1928). The possibility of a protective phagocytosis in virus diseases suggests itself, but the term *fixation of virus* has been employed here because it was thought to cover the various possibilities of adsorption on to the cells, phagocytosis by the cells and an active penetration of the cells by the virus.

The work of Andrewes (1928) on vaccinia supports the view that no destructive action of antibodies on virus occurs *in vitro*, the experiments recorded here suggest that neither does such action occur directly *in vivo*. The simultaneous presence of virus and antibodies in the circulation over a period of several days might however possibly be explained on the assumption that the virus is protected from a destructive action of antibodies by its intra-cellular situation. Against this view is the fact that the blood-cells of a solidly immune animal take up virus inoculated intravenously as a prelude to its rapid disappearance from the circulation. This indicates that such cells play an important part in the phenomena of immunity, whether they kill the virus *in situ* or migrate from the blood-stream so that the process of destruction may occur elsewhere it is at present impossible to say.

The appearance of antibodies in the blood as early as the second day after infection suggests that in the collection of skin scrapings or other tissues for the preparation of vaccines a preliminary exsanguination of the animal might prove advantageous.

SUMMARY.

1. Vaccinia virus inoculated into the circulation of a rabbit is rapidly fixed by some of the formed elements of the blood. The same holds true of virus which invades the blood-stream after intradermal infection.

2. A fractionation technique is described by which virus may be demonstrated in suspensions of washed cells although none is apparent when the whole blood is tested. By use of this method virus was recovered from the blood constantly up to 8 days after infection.

3. The white blood-cells are probably responsible for this virus fixation, but the platelets have not yet been definitely excluded. The red blood-corpuscles play no part in the process.

4. Virus inoculated intravenously into an immune rabbit is likewise taken up by the formed elements of the blood, but quickly disappears from the circulation (4–6 hours).

5. The masking of virus in whole blood is shown to be due to the early appearance of antibodies in the plasma. Such antibodies may appear as early as the second day after infection and increase up to the sixth or seventh day.

6. Virus and antibodies may exist simultaneously in pathological exudates due to vaccina. The virus is closely associated with the cellular constituents, the antibodies with the liquid.

In conclusion I wish to express my indebtedness to the Director of this Department, Capt. S. R. Douglas, for his helpful suggestions and constant advice.

REFERENCES.

ANDREWES, C. H.—(1928) *J. Path. Bact.*, **31**, 671.
CALMETTE, A., ET GUERIN, C.—(1901) *Ann. Inst. Pasteur*, **15**, 161.
DOUGLAS, S. R., SMITH, W., AND PRICE, L. R. W.—(1929) *J. Path. Bact.*, **32**, 99.
DAUBNEY, R.—(1928) *J. Comp. Path. Therap.*, **41**, 228.
FLEMING, A.—(1926) *Brit. J. Exp. Path.*, **7**, 281.
GILDEMEISTER, E., AND HEUER, G.—(1928) *Zbl. Bakt.*, Abt. I, Orig., **106**, 58.
GOLDMAN, A.—(1928) *Ibid.*, Abt. I, Orig., **105**, 333.
HOEN, E., TSCHERTKOW, L., UND ZIPP, W.—(1926) *Z. Hyg. InfektKr.*, **106**, 624.
LEVADITI, C., ET NICOLAU, S.—(1923) *C. R. Acad. Sci.*, Paris, **77**, 466.
NICOLLE, C., CONOR, A., AND CONSEIL, E.—(1912) *Ann. Inst. Pasteur*, **26**, 250.
OHTAWARA, T.—(1922) *Sci. Rep. Govt. Inst. Infect. Dis.*, Tokyo, **1**, 203.
RIVERS, T. M., AND TILLETT, W. S.—(1923) *J. Exp. Med.*, **38**, 673.
TODD, C.—(1928) *Brit. J. Exp. Path.*, **9**, 19.
Idem, AND WHITE, R. G.—(1914) 'Experiments on Cattle Plague.' Cairo (Government Press).
WALTHARD, B.—(1927) *Z. Hyg. InfektKr.*, **107**, 221.

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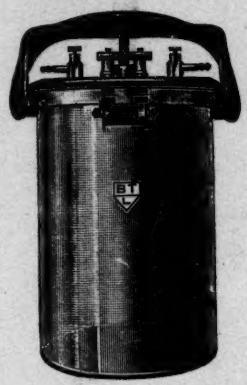

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